BM

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 January 2003 (09.01.2003)

PCT

(10) International Publication Number
WO 03/003009 A1

(51) International Patent Classification[7]: G01N 33/50

(21) International Application Number: PCT/DK02/00456

(22) International Filing Date: 28 June 2002 (28.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PA 2001 01027	29 June 2001 (29.06.2001)	DK
PA 2001 01028	29 June 2001 (29.06.2001)	DK
PA 2001 01030	29 June 2001 (29.06.2001)	DK
PA 2001 01031	29 June 2001 (29.06.2001)	DK
60/301,931	29 June 2001 (29.06.2001)	US
PA 2001 01026	29 June 2001 (29.06.2001)	DK
PCT/DK01/00867	21 December 2001 (21.12.2001)	DK

(71) Applicant *(for all designated States except US)*: **7TM PHARMA A/S** [DK/DK]; Rønnegade 2, DK-2100 Copenhagen Ø (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **RIST, Øystein** [NO/DK]; Vestervigvej 6, DK-2720 Vanløse (DK). **HÖGBERG, Thomas** [SE/SE]; Kabbarpsvägen 6, SE-232 52 Akarp (SE). **HOLST LANGE, Birgitte** [DK/DK]; Ved Klosteret 2, DK-2100 Copenhagen Ø (DK). **SCHWARTZ, Thue W.** [DK/DK]; Steen Blichersvej 13, DK-2000 Frederiksberg (DK). **ELLING, Christian, E.** [DK/DK]; Fredensvej 45, DK-2970 Hørsholm (DK).

(74) Agent: **ALBIHNS A/S**; H.C. Andersens Boulevard 49, DK-1553 Copenhagen (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF METAL-ION CHELATES IN VALIDATING BIOLOGICAL MOLECULES AS DRUG TARGETS IN TEST ANIMAL MODELS

(57) Abstract: Use of chemical compounds or selections of chemical compounds (libraries) of the general Formula (I): for *in vivo* methods for testing or validating the physiological importance and/or the therapeutic or pharmacological potential of biological target molecules, notably proteins such as, e.g., receptors and especially 7TM receptors in test animals expressing the biological target molecule with, notably, a silent, engineered metal-ion site. Use of specific metal-ion binding sites of a generic nature in specific biological target molecules such as, e.g. transmembrane proteins wherein the metal-ion binding site is capable of forming a complex with a metal ion is also described. Chemical compounds or libraries suitable for use in methods for improving the *in vivo* pharmacokinetic behaviour of metal-ion chelates (e.g. the absorption pattern, the plasma half-life, the distribution, the metabolism and/or the elimination of the metal-ion chelates). In order to improve the efficacy of the metal-ion chelates impact on the biological target molecule after administration of the metal-ion chelate *in vivo* to a test animal it is advantageous e.g. to increase the time period during which the metal-ion chelate is in the circulatory system and/or localised at the target. Metal-ion chelating compounds, which are designed to be suitable for use in a target validation process according to the invention and to libraries of at least two or more of such metal-ion chelating compounds are disclosed.

WO 03/003009 A1

USE OF METAL-ION CHELATES IN VALIDATING BIOLOGICAL MOLECULES AS DRUG TARGETS IN TEST ANIMAL MODELS

Field of the invention

The present invention relates to the use of chemical compounds or selections of chemical compounds (libraries) for *in vivo* methods for testing or validating the physiological importance and/or the therapeutic or pharmacological potential of biological target molecules, notably proteins such as, e.g., receptors and especially 7TM receptors in test animals expressing the biological target molecule with, notably, a silent, engineered metal-ion site.

The present invention also relates to the use of specific metal-ion binding sites of a generic nature in specific biological target molecules such as, e.g. transmembrane proteins wherein the metal-ion binding site is capable of forming a complex with a metal ion.

A test animal suitable for use in the present invention is normally a genetically modified animal. At any given time during the development of the test animal or in adult life, it is then possible to turn the biological target molecule (such as, e .g., the receptor) on or off – depending on the engineered site with a pharmacological tool, i.e. a metal-ion chelate formed between a metal-ion and a metal-ion chelator. The thus developed pharmacologically controlled "knock-out" methods are useful in the evaluation of biological target molecules such as, e.g., proteins as drug targets as well as in the characterization of the physiological role of orphan receptors.

The present invention also relates to chemical compounds or libraries suitable for use in methods for improving the *in vivo* pharmacokinetic behaviour of metal-ion chelates (e.g. the absorption pattern, the plasma half-life, the distribution, the metabolism and/or the elimination of the metal-ion chelates). In order to improve the efficacy of the metal-ion chelates impact on the biological target molecule after administration of the metal-ion chelate *in vivo* to a test animal it is advantageous e.g. to increase the time period during which the metal-ion chelate is in the circulatory system and/or localised at the target.

Notably, the invention relates to metal-ion chelating compounds, which are designed to be suitable for use in a target validation process according to the invention and to libraries of at least two or more of such metal-ion chelating compounds.

Background of the invention

During the last decades drug discovery methods have emerged involving *in vitro* testing of libraries of chemical compounds for interaction with possible drug targets in order to determine one or more lead compounds which are drug substances or potential starting compounds for the development of a novel drug substance. In continuation of such developmental work the substances should be tested in an *in vivo* animal model.

Furthermore, the drug targets known today are only a small fraction of the huge number of potential drug targets that are currently becoming available through the characterization of the humane genome. Even for subtypes of well-known receptors (e.g. monoamine receptor and neuropeptide receptor subtypes) we do not know the physiological function and/or the pharmacological potential thereof. Accordingly, it is a difficult task to evaluate new drug targets since our knowledge of their physiological role normally is very limited.

Moreover, problems that have limited the use of drug discovery means and methods are the lack of a reliable validation method of a discovered drug or lead substance and/or a proper validation method of a specific target. As will be explained in the following, the known methods for *in vivo* target validation all have some important disadvantages.

The subject of the present invention is methods for target validation, i.e. for validating the function of a specific target or for validating the effect of specific substances acting on that target. The present invention is also directed to how to design and develop chemical compounds suitable for use in such methods.

Classical pharmacological knock-out methods

One technology, which has been employed for evaluating drug targets in general, has been various forms of gene knock-out methods. Using such methods it is possible to specifically delete the gene for a particular biological target molecule from the genome of a test animal – usually a mouse. It is also possible by transgenic techniques to over-express a particular receptor gene in a tissue specific manner. From the phenotype of such animals the usefulness of a particular receptor as a drug target is then evaluated. In many cases the phenotype of a receptor knock-out animal corresponds to what would be expected based on the effect of known drugs, acting through the receptors in question.

However, the method is not always working as expected. For example, deletion of certain genes causes unforeseen problems in being embryonic lethal or inducing developmental malformations. Moreover, it is often seen when the classical knock-out technology is employed in a well-characterized gene system, that the transgenic animals develop compensatory mechanisms, which impair the interpretation of the phenotype. Thus, the deletion of one gene may lead to the up-regulation of other redundant regulatory systems that masks the effect of the gene knockout, i.e. the suspected effect of a putative or antagonistic drug in the animal model is masked.

Considering the weakness of the classical knockout methods a number of other methods are currently being developed.

Conditional pharmacological knock-out methods

Conditional pharmacological knock-out is a recently developed technique by which the expression is turned off in an inducible manner, both in respect of tissue and time. The crucial difference from the classical knockout method is that two different genetic modifications have to be introduced, which accordingly require development of two different transgenic animals. One of the genetic modifications is introduction of an artificial gene fragment, which codes for a *recombinase* enzyme. The promoter of this gene element obtains cell type specificity. Whereas the time dependence is achieved by use of an inducible promoter, that require an *inducer* (e.g. tetracycline) in order to become active. The other genetic modification includes introduction of a silent gene fragment, e.g. loxP, upstream of the relevant gene, which addresses the *recombinase* enzyme very specifically. The recombination event destroys the adjacent gene and turn off the expression. The main disadvantages are:

1. it is only possible to inhibit the gene expression partially with about 25% expression remaining.
2. inhibition of the gene product is not a direct consequence of the administration of the *inducer*, as is seen with administration of ordinary drug that target the protein directly. In contrast it takes a while - often several days - from administration of the *inducer* to the inhibition of the protein expression of the protein level is obtained.
3. two different transgenic animals have to be developed, one expressing the transactivator gene and the other expressing the silent gene upstream of the relevant gene.

(Li-Na Wei et al., (1997) Ann rev Pharm Tox 37: 119-141)

Antisense techniques

The antisense technique is based on a specific base-pair interaction between the target gene, frequently at messenger RNA level, and the antisense probe. This hybrid is subsequently degraded by endogenous double strand specific ribonucleases, hence, no translation or protein expression will occur. It is also possible to address the chromatin DNA with the antisense probe forming triple helices and thereby stop the transcription. *In vivo* the gene is administered either by transgenic expression of the gene under an inverted promoter, by viruses or by direct injection of the nucleotide fragment. Often recognised disadvantages of the antisense techniques include:

1. Unpredictable degradation of the antisense probes,
2. Optimising the region and the length of the antisense homology can only be determined by trial and error, which is very time consuming.
3. Problems related to bioavailability, distribution and pharmacokinetics.

(James A. H. Murray, (1994) Antisense RNA and DNA, Modern cell Biology Wileyliss)

Specifically designed Zinc finger proteins

This technology utilizes engineered zinc finger DNA-binding proteins (ZFPs) to regulate gene expression. The ZFP's have two different domains: a DNA binding domain and a functional domain, the later may have either activating or repressing abilities. By substitution of amino acids in the DNA binding domain it is possible to generate ZFP that recognize and bind to specific DNA sequences, and thereby turn off or on the adjacent gene (Rebar, E. J. & Pabo, C. O (1994); Science 263:671).

All the above-mentioned known methods have their advantages – but also their drawbacks. Mainly they mimic poorly a true pharmacological setting. Furthermore, in the light of the recent advances in the field of drug discovery there is an increasing need for developing highly efficient, simple and effective methodologies and means for performing *in vivo* validation of the pharmacological potential and physiological importance of biological target molecules and substances interacting with such proteins.

The present invention aims at fulfilling this need. The present invention provides a novel target validation method using test animals, which express biological target molecules such as, e.g., proteins like receptors with engineered metal-ion sites. It is important to note that metal-ion sites may be constructed as silent "switches", i.e. in a way that allows the natural ligand to bind normally in the absence of the metal ion. However, in the

presence of the metal ion or rather metal-ion chelate, the activity of the biological target molecule is inhibited or stimulated. If the wild-type protein in an animal is replaced with a protein holding a silent metal ion switch, the animal should develop normally and no compensatory mechanisms would be up-regulated in the absence of the metal ion. First at the moment when a metal ion, e.g. in the form of a metal-ion chelate is administrated to the test animal the switch is turned on or off and the physiological and/or pharmacological impact on the animal can be monitored.

Summary of the invention

The present invention relates to chemical compounds for use in a target validation process for testing or validation the physiological importance and/or the therapeutic potential of a biological target molecule.

The process comprising

i) introduction of a silent metal ion site in the biological target molecule to obtain a silent metal ion engineered biological target molecule,

ii) *in vitro* testing of a test compound for its ability to bind to the introduced silent metal ion site in the silent metal ion engineered biological target molecule or for any change in the activity of the biological target molecule,

iii) optionally, chemically optimising the test compound and/or the biological target molecule to create secondary interaction(s), especially by forming covalent or slowly reversible bonds with –SH, OH or NH_2 groups in the vicinity of the metal ion site in the silent metal ion engineered biological target molecule or by forming ionic interactions with charged groups such as Asp, Glu, Lys, and Arg. Optionally the secondary interaction residues present in e.g. Cys, Ser, Thr, Tyr, His, Asp, Glu, Lys, and Arg can be introduced by engineering into the biological target in the vicinity of the silent metal ion binding site,

iv) optionally, repeating any of steps ii) and iii) to obtain a suitable binding affinity in the *in vitro* test,

v) optionally, chemically optimising the test compound to improve the pharmacokinetic and/or biopharmaceutical properties of the test compound,

vi) preparing a genetically modified test animal containing the silent metal ion site engineered biological target molecule,

vii) *in vivo* testing of the optionally optimised test compound in the genetically modified test animal, and monitoring the biochemical, physiological and/or behaviour parameters of the genetically modified test animal.

The present invention also relates to the use of specific metal-ion sites in specific biological target molecules in a target validation process

Furthermore, the invention relates to the use of test compounds of formula I, to libraries comprising such compounds and to the use of such libraries in a target validation process.

Detailed disclosure of the invention

As mentioned above, the present invention relates to the use of chemical compounds for use in methods for *in vivo* target validation, i.e. methods to determine the effect of a specific agonist or antagonist for a specific biological target molecule. As agonist or antagonist metal-ion chelates are used Elling et al. (*PNAS 96*, 1999, pp. 12322-12327) and Holst et al. (Mol. Pharmacol. 2000, 58: 263-270) have indicated a use of metal-ion chelators for such purposes but considered the affinity of the metal ion sites to be too low. However, the present invention provides means for establishing a suitable affinity and a suitable pharmacokinetic behaviour.

The present invention is based on a technology which makes it possible to genetically modify animals in such a manner that they express a silent metal-ion site in a potential drug target, i.e. a metal-ion site is created in which the mutations do not significantly affect the binding and action of the endogenous ligand for the biological target molecule such as, e.g., a receptor. When such a metal-ion site engineered biological target molecules (e.g. a receptor) is introduced into an animal by classical gene-replacement technology, i.e. exchange of the gene coding for the endogenous biological target molecule with the gene coding for the metal-ion site engineered biological target molecule, then the animals will develop normally without compensatory mechanisms, which otherwise frequently impair the interpretation of the phenotypes of the animals in classical gene knock-out technology. As explained above, in the adult animals or whenever it is found appropriate the animals are then treated with an appropriate metal-ion chelating agent, which then will

act as an antagonist (or agonist) and turn off (or on) the function of the metal-ion site engineered biological target molecules such as, e.g. a receptor. Before the present invention was made, such an approach was impaired by the fact, that the generally available metal-ion chelating agent only will bind with at best μM affinity and with a rather poor selectivity to the metal-ion site engineered biological target molecule, which will give similar μM or lower antagonistic potencies. These relatively low potencies and the relative low specificity of such metal ion chelating agents impairs the general applicability of the technology due to simple pharmacokinetic and toxicological problems. With the technology of the present invention it is possible to increase the affinity of metal-ion chelates significantly and make them more stable, which will make it considerably more easy to reach therapeutic and efficient concentrations of the metal-ion chelates in the animals and also to increase the "therapeutic window" due to the higher degree of selectivity of the small organic molecule ligands (i.e. the metal ion chelates which also are denoted test compounds) caused by the establishment of more than one molecular interaction point. Establishment of just a single or a few additional secondary chemical interaction(s), e.g. a charge-charge interaction, will increase the affinity and selectivity of the metal-ion chelate significantly and thereby making the whole process usable. In a preferred embodiment of the invention, a specific covalent bond is introduced between the metal-ion chelate and a residue in the biological target molecule.

As mentioned above, a target validation process is performed by establishing an interaction between a silent metal ion site in a biological target molecule and a chelate. To this end it is important to note that the chelate suitable for use according to the present invention may be a test compound i.e. a small organic compound itself or it may be a transport form or a depot form of the test compound provided that the function of the test compound is not significantly impaired, i.e. it is able to interact with the silent metal ion site and possible secondary interaction site(s) on the biological target molecule. Although the test compound itself may be very effective in establishing an interaction between the target molecule and itself, an improvement of its pharmacokinetic properties or its biopharmaceutical properties may be required in order to e.g. make sure that the test compound reaches the site of action or is maintained in the circulatory system long enough to give rise to a physiological effect.

A target validation process employing chemical compounds or libraries according to the invention includes the following main steps:

1. a process in which a silent metal-ion site is introduced into a biological target molecule
2. an *in vitro* test in which a test compound is tested for its ability to bind to the engineered metal ion site in the biological target molecule
3. optionally, a chemical optimisation step in which the test compound and/or the biological target molecule is modified in such a manner that secondary interactions with chemical groups in the vicinity of the metal ion site in the biological target molecule are created
4. optionally, repetition of step(s) 2 (and 3) until a suitable binding affinity is obtained,
5. optionally, chemical optimisation of the test compound in order to improve the pharmacokinetic and/or biopharmaceutical properties of the test compound,
6. preparation of a genetically modified test animal in which the metal-ion site engineered biological target molecule is introduced into the animal,
7. *in vivo* testing of the thus optimised test compound in test (normally together with testing of control animals), and monitoring the biochemical, physiological and/or behaviour parameters of the test (and control animals), and
8. optionally, repetition of one or more of the above-mentioned steps.

In general, step 3 mentioned above is necessary in the target validation process according to the invention. However, in those cases where the test compound itself has a structure which enables secondary interaction(s) to take place, or the test compound already has been optimised according to step 3 above and fall within the definition of test compounds given herein, step 3 may be irrelevant and can thus be excluded.

In the following these steps will be discussed in detail.

Definitions

Throughout the text including the claims, the following terms shall be defined as indicated below.

A *"chemical compound"* or a *"test compound"* is intended to indicate a small organic molecule ligand or a small organic compound, which is capable of interacting with a biological target molecule, in particular with a protein, in such a way as to modify the biological activity thereof. The term includes in its meaning metal ion chelates of the formulas shown below. Furthermore, the term includes in its meaning metal ion chelates of the formulas shown below as well as chemical derivatives thereof constructed to

interact with other part(s) of the biological target molecule than the metal ion binding site. A test compound may also be an organic compound, which in its structure includes a metal atom via a covalent binding.

A *"metal ion chelator"* is intended to indicate a compound capable of forming a complex with a metal atom or ion, and contains at least two interactions between the metal centre and the chelator. Such a compound will generally contain two heteroatoms such as N, O, S, Se or P with which the metal atom or ion is capable of forming a complex.

A *"ligand"* is intended to indicate a functional group or structural element in a molecule that binds/coordinates a metal ion.

A *"metal ion chelate"* is intended to indicate a complex of a metal ion chelator and a metal atom or ion.

A *"metal ion"* is intended to indicate a charged or neutral element. Such elements belong to the groups denoted main group metals, light metals, transition metals, semi-metals or lanthanides (according to the periodic system). The term "metal ion" includes in its meaning metal atoms as well as metal ions.

A *"library"* is intended to indicate a collection of chemical compounds having a common basic structural element. The number of compounds in a library is three or more. All the chemical compounds contained in a library according to the invention have the same common basic structural element or scaffold. The number of compounds in a library is generally in a range from about 3 to about 250 such as, e.g. from about 3 to about 100, from about 3 to about 50, from about 3 to about 30, from about 3 to about 25, from about 3 to about 20, from about 3 to about 15 or from about 3 to about 10 such as at least 3, 4, 5, 6, 7, 8, 9 or 10 compounds. More generally the number of compounds in a library is in a range of from about 3 to about 10,000 compounds such as, e.g. from about 5 to about 5,000, from about 10 to about 2,500, from about 3 to about 1,000, from about 3 to about 750, from about 3 to about 500, from about 3 to about 250, from about 3 to about 100, from about 3 to about 75, from about 3 to about 50, from about 3 to about 25, from about 3 to about 10 compounds. In general, libraries based on focused structures contain from about 3 to about 500 compounds such as, e.g. from about 3 to about 100 compound, whereas chemical diverse randomized libraries contain from about 500 to about 10,000 compounds such as, e.g. 750 to about 10,000 compounds, from about 1,000 to about 10,000 compounds.

A *"metal-ion binding site"* is intended to indicate a part of a biological target molecule, which comprises an atom or atoms capable of complexing with a metal atom or ion. Such an atom will typically be a heteroatom, in particular N, O, S, Se or P. With respect to proteins a metal-ion binding site is typically an amino acid residue of the protein, which comprises an atom capable of forming a complex with a metal ion. These amino acid residues are typically, but not restricted to, histidine, cysteine, glutamate and aspartate.

A *"receptor-ligand"* is intended to include any substance that either inhibits or stimulates the activity of a biological target molecule such as, e.g. a protein or that competes for a receptor in a binding assay. An "agonist" is defined as a ligand increasing the functional activity of a biological target molecule (e.g. signal transduction through a receptor). An "antagonist" is defined as a ligand decreasing the functional activity of a biological target molecule either by inhibiting the action of an agonist or by its own intrinsic activity. An "inverse agonist" (also termed "negative antagonist") is defined as a receptor-ligand decreasing the basal functional activity of a biological target molecule.

A *"biological target molecule"* is intended to include proteins such as, e.g., membrane proteins, nucleic acids, carbohydrates, nucleoproteins, glycoproteins and glycolipids. In the present context the biological target molecule normally has been manipulated to contain a metal-ion binding site. However, in some cases, the biological target molecule may be in its wild-type form.

A *"protein"* is intended to include any protein, polypeptide or oligopeptide with a discernible biological activity in any unicellular or multicellular organism, including bacteria, fungi, plants, insects, animals or mammals, including humans. Thus, the protein may suitably be a drug target, i.e. any protein which activity is important for the development or amelioration of a disease state, or any protein which level of activity may be altered (i.e. up- or down-regulated) due to the influence of a biologically active substance such as a small organic chemical compound.

A *"membrane protein"* is intended to include but is not limited to any protein anchored in a cell membrane and mediating cellular signalling from the cell exterior to the cell interior. Important classes of membrane proteins include receptors such as tyrosine kinase receptors, G-protein coupled receptors, adhesion molecules, ligand- or voltage-gated ion channels, or enzymes. The term is intended to include membrane proteins whose function is not known, such as orphan receptors. In recent years, largely as part of the human

genome project, large numbers of receptor-like proteins have been cloned and sequenced, but their function is as yet not known. The present invention may be of use in elucidating the function of the presumed receptor proteins by making it possible to develop methods of identifying ligand for orphan receptors based on compounds developed from metal ion chelates that bind to mutated orphan receptors into which artificial metal ion binding sites have been introduced.

"Signal transduction" is defined as the process by which extracellular information is communicated to a cell by a pathway initiated by binding of a ligand to a membrane protein, leading to a series of conformational changes resulting in a physiological change in the cell in the form of a cellular signal.

A *"functional group"* is intended to indicate any chemical entity which is a component part of the test compound and which is capable of interacting with an amino acid residue or a side chain of an amino acid residue of the biological target molecule such as, e.g. a membrane protein. A functional group is also intended to indicate any chemical entity which is a component part of the biological target molecule and which is capable of interacting with other parts of the biological target molecule or with a part of the test compound. Examples of such functional groups include, but are not limited to, ionic groups involved in ionic interactions such as e.g. the ammonium ion or carboxylate ion; hydrogen bond donor or acceptor groups such as amino, amide, carboxy, sulphonate, etc.; and hydrophobic groups involved in hydrophobic interactions, pi-stacking, ion-dipole interactions, dipole-dipole interactions, edge-on aromatic interactions, dispersion and induction forces, metal complex interactions and the like.

A *"wild-type"* membrane protein is understood to be a membrane protein in its native, non-mutated form, in this case not comprising an introduced metal-ion binding site or residues introduced for secondary interactions.

The term *"in the vicinity of"* is intended to include an amino acid residue or any other residue or functional group located in the area defining the binding site of the metal ion chelate and at such a distance from the metal ion binding amino acid residue that it is possible, by attaching suitable functional groups to the test compound, to generate an interaction between said functional group or groups and said amino acid residue, another residue or functional group.

A *"silent metal ion site"* is intended to mean a metal-ion site engineered into a biological target molecule in such a manner, that it does not affect or significantly does not affect the function of the biological target molecule, i.e. in the case of a receptor, for example the binding and function of the endogenous ligand. The ultimate test for degree of silence will be performed *in vivo* in the genetically modified animals. However, it is expected that a certain degree of up-regulation of the endogenous ligand will occur in the fine-tuning of the signalling system, which can compensate for a certain degree of loss-of-function due to the metal-ion engineering. This should not be confused with up-regulation of other signalling pathways etc., which frequently occurs when the receptor is totally missing as in a real knock-out animal. A silent metal ion site may be evaluated in an *in vitro* model employing transiently transfected cultured cell systems measuring, for example binding affinities and functional responses. In the present context, a metal ion site is considered to be a silent metal ion site if it does not change the structure and function of the biological target molecule at all or only change its structure and function to a limited extent. For example, in case the biological target molecule is a cell surface receptor, a metal-ion site will be considered to be silent if no or only limited change in the cellular surface expression of the receptor occurs and/or if no or only limited change in binding affinity of the endogenous ligand – being a hormone, a transmitter or another chemical messenger - is observed and no or only limited change is observed in the ability of the endogenous ligand to stimulate signal transduction through the receptor. As determined in *in vitro* cell expression systems no change in the cellular surface expression and no change in the affinity of the receptor are preferred. However, up to 3-5 fold decrease in either surface expression or affinity is expected to be tolerated and considered to be "silence". In some cases up to 8-10 fold decrease in surface expression or affinity may be tolerated and in certain cases even up to 20 fold decrease or more in surface expression or affinity could perhaps be tolerated. To test for cell expression and affinity for the endogenous ligand various forms of standard *in vitro* expression systems for the biological target molecule can be used. For example, transfection of tissue culture cells with expression plasmids ensuring a suitable, controllable gene expression in a cell type where the biological target molecule is expressed in a cellular context being as close as possible to the cellular contexts in which the biological target molecule normally is expressed *in vivo*.

A *"genetically modified animal"* means an animal in which a chromosome or a part thereof has been modified so that a specific gene or gene sequence has been deleted, exchanged with another or a further gene or gene sequence has been inserted. In the present context a genetically modified animal specifically refers to an animal in which a gene coding for a biological target molecule has been introduced in a modified form which

makes it suitable for target validation though treatment of the animal with a receptor-ligand, which specifically affects the function of the modified gene product. It is also possible to introduce a gene modification in the test animal in its embryonic state. Preferably the gene is introduced into the animal by selectively replacing the gene coding for the endogenous biological target molecule, however this is not a requirement.

A *"test animal"* is intended to embrace a genetically modified animal as well as a non-genetically modified animal. Suitable test animals for use in methods according to the invention are mammals such as, e.g., rodents, mice, rats, rabbits, guinea pigs, monkeys, dogs, domestic animals such as, e.g. pigs, cows, horses racing animals such as, e.g., horses, dogs.

A *"spacer"* or a "linker" is intended to embrace bifunctional chemical substances which on the one hand are able to react or interact with the test compound and on the other hand with a carrier.

A *"carrier"* is intended to embrace compounds that provides the test compounds with specific properties, e.g. with respect to the physiological, pharmacokinetic and/or biopharmaceutic behaviour of the test compound. The carrier is normally directly or indirectly linked to the test compound through establishment of a covalent or a non-covalent bound. When the carrier is indirectly linked to the test compound, a spacer between the test compound and the carrier is normally included.

Nature of the biological target molecules

The biological target molecules include but are not restricted to proteins, nucleoproteins, glycoproteins, nucleic acids, carbohydrates, and glycolipids. In the present context the biological target molecule normally has been genetically manipulated to contain a metal-ion binding site, but in some cases the biological target molecule may *per se* contain a metal-ion binding site. Irrespective of whether the metal-ion binding site has been artificially introduced or it is a natural site, the metal ion site is preferably silent. In preferred embodiments the biological target molecule is a protein, which may be for example a membrane receptor, a protein involved in signal transduction, a scaffolding protein, a nuclear receptor, a steroid receptor, a transcription factor, an enzyme, and an allosteric regulator protein, or it may be a growth factor, a hormone, a neuropeptide or an immunoglobulin.

Proteins as drug targets

Most drug compounds act by binding to and altering the function of proteins. These can be intracellular proteins such as, for example enzymes and transcription factors, or they can be extracellular proteins, for example enzymes, or they can be membrane proteins. Membrane proteins constitute a numerous and varied group whose function is either structural, for example being involved in cell adhesion processes, or the membrane proteins are involved in intercellular communication and communication between the cell exterior and the interior by transducing chemical signals across cell membranes, or they facilitate or mediate transport of compounds across the lipid membrane. Membrane proteins are for instance receptors and ion channels to which specific chemical messengers termed ligands bind resulting in the generation of a signal, which gives rise to a specific intracellular response (this process is known as signal transduction). Membrane proteins can, for example also be enzymes, which are associated to the membrane for functional purposes, e.g. proximity to their substrates. Most membrane proteins are anchored in the cell membrane by a sequence of amino acid residues, which are predominantly hydrophobic to form hydrophobic interactions with the lipid bilayer of the cell membrane. Such membrane proteins are also known as integral membrane proteins. In most cases, the integral membrane proteins extend through the cell membrane into the interior of the cell, thus comprising an extracellular domain, one or more transmembrane domains and an intracellular domain.

A large fraction of current drugs act on membrane proteins and among these the majority are targeted towards the G protein coupled receptors (GPCR) with their seven transmembrane segments, also called 7TM receptors.

Membrane proteins as drug targets

In particularly preferred embodiments the biological target molecule is a membrane protein, which suitably is an integral membrane protein, which is to say a membrane protein anchored in the cell membrane. The membrane protein is preferably of a type comprising at least one transmembrane domain. Interesting membrane proteins for the present purpose are mainly found in classes comprising 1-14 transmembrane domains.

1TM - membrane proteins of interest comprising one transmembrane domain include but are not restricted to receptors such as tyrosine kinase receptors, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming

growth factor, erythropoietin, colony-stimulating factor, platelet-derived growth factor receptor or nerve growth factor receptor (TrkA or TrkB).

2TM - membrane proteins of interest comprising two transmembrane domains include but are not restricted to, e.g., purinergic ion channels.

3, 4, 5TM - membrane proteins of interest comprising 3, 4 or 5 transmembrane domains includes but are not restricted to e.g. ligand-gated ion channels, such as nicotinic acetylcholine receptors, GABA receptors, or glutamate receptors (NMDA or AMPA).

6TM - membrane proteins of interest comprising 6 transmembrane domains include but are not restricted to e.g., voltage-gated ion channels, such as potassium, sodium, chloride or calcium channels.

7TM – the membrane protein of interest comprising a G-protein coupled receptor, such as the receptor for (– in brachet the receptor subtypes are mentioned): acetylcholine (m1-5), adenosine (A1-3) and other purines and purimidines (P2U and P2Y1-12), adrenalin and noradrenalin (α1A-D, α2A-D and β1-3), amylin, adrenomedullin, anaphylatoxin chemotactic factor, angiotensin (AT1A, -1B and -2), apelin, bombesin, bradykinin (1and 2), C3a, C5a, calcitonin, calcitonin gene related peptide, CD97, conopressin, corticotropin releasing factor (CRF1and -2), calcium, cannabinoid (CB1and -2), chemokines (CCR1-11, CXCR1-6, CX3CR and XCR), cholecystokinin (A-B), corticotropin-releasing factor (CRF1-2), dopamine (D1-5), eicosanoids, endothelin (A and B), fMLP, Frizzled (Fz1,2,4,5 and 7-9), GABA (B1 and B2), galanin, gastrin, gastric inhibitory peptide (glucose-dependent insulinotropic polypeptide), glucagon , glucagon-like peptide I and II, glutamate (1-8), glycoprotein hormone (e.g. FSH, LSH, TSH, LH), growth hormone releasing hormone, growth hormone secretagogue /Ghrelin, histamine (H1-4), 5-hydroxytryptamine (5HT1A-1F, -2A-C and -4-7), leukotriene, lysophospholipid (EDG1-4), melanocortins (MC1-5), melanin concentrating hormone (MCH 1 and 2), melatonin (ML1A and 1B), motilin, neuromedin U, neuropeptide FF (NFF1and 2), neuropeptide Y (NPY1,2,4,5 and 6), neurotensin (1 and 2), nocioceptin, odor components , opiods (κ, δ, μ and x), orexins(OX1and -2) , oxytocin, parathyroid hormone/parathyroid hormone-related peptides, pheromones, platelet-activating factor, prostaglandin (EP1-4 and F2) prostacyclin, pituitary adenylate activating peptide, retinal, secretin, smoothernd, somatostatins (SSTR1-5), tachykinins (NK1-3), thrombin and other proteases acting through 7TM receptor, thromboxane, thyrotropin-releasing hormone, vasopressin (V1A, -1B and -2), vasoactive intestinal peptide, urotensin II, and virally encoded receptors

(US27, US28, UL33, UL78, ORF74, U12, U51); and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned such as mas-proto-oncogene, EBI (I and II), lactrophilin, brain specific angiogenesis inhibitor (BAI1-3), EMR1, RDC1 receptor, GPR12 receptor or GPR3 receptor, and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned.

8, 9, 10, 11, 12, 13, 14TM - Membrane proteins of interest comprising 8 to 14 transmembrane domains include but are not restricted to e.g., transporter proteins, such as, e.g., i) Na^+ cotransporters, including Na^+,Cl^- transporters, such as, e.g., GABA transporters, monoamine transporters, neutral amino acids transporters, kreatinin transporters and nucleoside transporters, and Na^+,K^+ coupled transporter such as, e.g., glutamate transporters, neutral amino acids transporters, and inositol transporters, and Na^+,glucose cotransporters, and Na^+,K^+,Cl^- cotransporters, ii) H+ coupled transporter including oligopeptide transporters and multi drug transporters, iii) antiporters, including Na^+/H^+- exchangers, anion exchangers such as, e.g., HCO_3^-/Cl^- exchangers and Na^+/Ca^+ exchangers, iv) ion-transporting ATPases including Na^+,K^+ ATPase, H^+,K^+ ATPase and Ca^{2+} ATPase and v) transporters from the ABC (ATP Binding Cassette) transporter family, including multidrug resistance related proteins and cystic fibrosis transmembrane regulators, and multidrug resistance proteins such as, e.g., P-glycoproteins, lung resistance related proteins and breast cancer resistance proteins.

The membrane protein may also be a cell adhesion molecule, including but not restricted to for example NCAM, VCAM, ICAM or LFA-1.

Furthermore, the membrane protein may be an enzyme such as adenylyl cyclase.

7TM overview

In a particularly preferred embodiment of the invention, the biological target molecules are 7 transmembrane domain receptors (7TM receptors) also known as G-protein coupled receptors (GPCRs). This family of receptors constitutes the largest super-family of proteins in the human body and a large number of current drugs are directed towards 7TM receptors, for example: antihistamines (for allergy and gastric ulcer), beta-blockers (for cardiovascular diseases), opioids (for pain), and angiotensin antagonists (for hypertension). These current drugs are directed against relatively few receptors, which have been known for many years. Today, several hundred 7TMs have been cloned and characterized, and the total number of different types of 7TMs in humans is presumed to

be between 1 and 2.000. The spectrum of ligands acting through 7TMs includes a wide variety of chemical messengers such as ions (e.g. calcium ions), amino acids (glutamate, γ-amino butyric acid), monoamines (serotonin, histamine, dopamine, adrenalin, noradrenalin, acetylcholine, cathecolamine, etc.), lipid messengers (prostaglandins, thromboxane, anandamide, etc.), purines (adenosine, ATP), neuropeptides (tachykinin, neuropeptide Y, enkephalins, cholecystokinin, vasoactive intestinal polypeptide, etc.), peptide hormones (angiotensin, bradykinin, glucagon, calcitonin, parathyroid hormone, etc.), chemokines (interleukin-8, RANTES, etc.), glycoprotein hormones (LH, FSH, TSH, choriogonadotropin, etc.) and proteases (thrombin). It is expected that a large number of the members of the 7TM superfamily of receptors will be suitable as drug targets. This notion is based on the fact that these receptors are involved in controlling major parts of the chemical transmission of signals between cells both in the endocrine and the paracrine system in the body as well as within the nervous system.

7TM receptor signalling - In 7TMs, binding of the chemical messenger to the receptor leads to the association of an intracellular G-protein, which in turn is linked to a secondary messenger pathway. The G-protein consists of three subunits, a α-subunit that binds and hydrolyses GTP, and a βγ-subunit. When GDP is bound, the α subunit associates with the βγ subunit to form an inactive heterotrimer that binds to the receptor. When the receptor is activated, a signal is transduced by a changed receptor conformation that activates the G-protein. This leads to the exchange of GDP for GTP on the α subunit, which subsequently dissociates from the receptor and the βγ dimer, and activates downstream second messenger systems (e.g. adenylyl cyclase). The α subunit will activate the effector system until its intrinsic GTPase activity hydrolyses the bound GTP to GDP, thereby inactivating the α subunit. The βγ subunit increases the affinity of the α subunit for GDP but may also be directly involved in intracellular signalling events.

7TM ligand-binding sites and silent metal-ion sites - Mutational analysis of 7TMs has demonstrated that functionally similar but chemically very different types of ligands can apparently bind in several different ways and still lead to the same function. Thus monoamine agonists appear to bind in a pocket relatively deep between TM-III, TM-V and TM-VI, while peptide agonists mainly appear to bind to the exterior parts of the receptors and the extracellular ends of the TMs (Strader et al., (1991) J. Biol. Chem. 266: 5-8; Strader et al., (1994) Ann. Rev. Biochem. 63: 101-132; Schwartz et al. Curr. Pharmaceut. Design. (1995), 1: 325-342). Moreover, ligands can be developed independent on the chemical nature of the endogenous ligand, for example non-peptide agonists or antagonists for peptide receptors. Such non-peptide antagonists for peptide receptors

often bind at different sites from the peptide agonists of the receptors. For instance, non-peptide antagonists may bind in the pocket between TM-III, TM-V, TM-VI and TM-VII corresponding to the site where agonists and antagonists for monoamine receptors bind (Holst et al. (1998) Mol.Pharmacol. 53:166-175). It has been found that in the substance P receptor, when the binding site for a non-peptide antagonist has been exchanged for a metal-ion binding site through introduction of His residues, no effect on agonist binding was observed (Elling et al., (1995) Nature 374: 74-77; Elling et al. (1996) EMBO J. 15: 6213-6219). Thus – metal-ion binding sites can be built which are silent for example in respect of interference with the binding of the endogenous ligand, which is important for the present invention of making silent metal-ion binding sites in biological target molecules.

Generic numbering system for 7TMs – The 7TM receptor superfamily is composed many hundreds of receptors that may be further divided into smaller sub-families of receptors. The largest of these smaller sub-families of 7TM receptors is composed of the rhodopsin-like receptors (also termed the family A receptors), which are named after the light-sensing molecule from our eye. The receptors are integral membrane proteins characterized by seven transmembrane (7TM) segments traversing the membrane in an antiparallel way, with the N-terminal on the extracellular side of the membrane and the C-terminal on the intracellular side. Within the membrane embedded part and in some cases in the membrane proximal parts, embedded in the aqueous environment surrounding the cell membrane, the polypeptide adopts a helical secondary structure. The lengths, and the beginning center and ends relative to the lipid bilayer membrane of these helices may be deduced from solved three-dimensional structures of the receptor proteins (Palczewski K. et al., Science, 289(5480), 2000, pp. 739-45). However, since the three-dimensional structure of only a single receptor has been solved to date, the helical lengths, and the beginning, center and ends relative to the lipid bilayer membrane of each of the seven helices may be dissected by sequence analysis (J.M. Baldwin, EMBO J. 12(4), 1993, pp. 1693-703; J.M. Baldwin et al., J. Mol Biol, 272(1), 1997, pp. 144-64).

A useful tool in the identification and engineering of metal-ion sites is the generic numbering system for residues of 7TM receptors. The largest family of 7TM receptors is composed of the rhodopsin-like receptors, which are named after the light-sensing molecule from our eye. Within the many hundred members of the rhodopsin-like receptor family, a number of residues, termed key residues, especially within each of the transmembrane segments are highly but not totally conserved. These residues may be used to direct an alignment of the primary protein sequences within the transmembrane

segments together with other standard principles and techniques, for example hydrophobicity plots, well-known to persons skilled in the art. Additionally, a number of other residues occur within the transmembrane segments that are very conserved, and these may be used to further direct an alignment of the transmembrane segments. These are particularly useful when a given key residue in a transmembrane segment has been substituted through evolution by another aminoacid of a dissimilar physiochemical nature.

However, due to differences in the length of especially the N-terminal segment, residues located at corresponding positions in different 7TM receptors are numbered differently in different receptors. However, based on the conserved key residues in each TM, a generic numbering system has been suggested (JM Baldwin, EMBO J. 12(4), 1993, pp. 1693-1703; TW Schwartz, Curr. Opin. Biotech. 5, 1994, pp. 434-444).
On the basis of the key residues present in the receptor family, the transmembrane segments are generically numbered. For example, in TM-II the highly conserved acidic function, aspartate (Asp) in the rhodopsin-like family is given the generic number 10, i.e. AspII:10, on the basis of its position in the helix. All other residues in the helix are hence numbered on this basis.

In Fig. 1 a schematic depiction of the structure of rhodopsin-like 7TMs is shown with one or two conserved, key residues highlighted in each TM: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17. In relation to the present invention it is important that residues involved in for example metal-ion binding sites can be described in this generic numbering system. For example, a tri-dentate metal-ion site constructed in the tachykinin NK1 receptor (Elling et al., (1995) Nature 374, 74-77) and subsequently transferred to the kappa-opioid receptor (Thirstrup et al., (1996) J. Biol. Chem. 271, 7875-7878) and to the viral chemokine receptor ORF74 (Rosenkilde et al., J. Biol. Chem. 1999 Jan 8; 274(2), 956-61) can be described to be located between residues V:01, V:05, and VI:24 in all of these receptors although the specific numbering of the residues is very different in each of the receptors. It is only in the rhodopsin-like receptor family that a generic numbering system has been established; however, it should be noted that although the sequence identity between the different families of 7TM receptors is very low, it is believed that they may share a more-or-less common seven helical bundle structure.

Hence an analogous system may be developed for the other families of 7TM receptors, for example the family B class of receptors, composed of for example the glucagon receptor, the glucagon-like peptide (GLP) receptor-1, the gastric inhibitory peptide receptor (GIP), the corticotropin releasing factor (CRF) receptor-1, vasoactive intestinal

peptide (VIP) receptor, pituitary adenylate cyclase-activating polypeptide (PACAP) receptor etc. (J.W. Tams et al., Receptors Channels 1998;5(2), pp. 79-90). Again, on the basis of the key residues present in the family B class of receptors, the transmembrane segments are generically numbered. For example, in TM-I, the highly conserved hydroxy function, serine (Ser) is given the generic number 8, i.e. SerI:08 on the basis of its position in the helix; in TM-II the highly conserved histidine (His) is given the generic number 6 i.e. HisII:06; in TM-III the highly conserved cysteine (Cys) is given the generic number 1 i.e. CysIII:01; in TM-IV the highly conserved proline (Pro) is given the generic number 13 i.e. ProIV:13; in TM-V the highly conserved asparagine (Asn) is given the generic number 14 i.e. AsnV:14; in TM-VI the highly conserved leucine (Leu) is given the generic number 9 i.e. LeuVI:09; in TM-VII the highly conserved glycine (Gly) is given the generic number 13 i.e. GlyVII:13. All other residues in the helices are hence numbered on this basis.

Thus, all the techniques described in the present invention can be applied to the other families of 7TM receptors with minor modifications. This generic numbering system together with general knowledge of the 3D structure of the 7TM receptors and knowledge from systematic metal-ion site engineering makes it possible to predict or identify the presence of metal-ion sites based on the DNA sequence coding for the 7TM receptor.

Orphan 7TM receptors – one embodiment of the invention is directed to a method of assessing the physiological function and pharmacological potential of orphan 7TM receptors by the introduction of metal-ion sites in the orphan receptor. During the cloning of 7TM receptors many "extra" receptors were discovered for which no ligand was known, the so-called orphan receptors. Today there are several hundreds of such orphan 7TM receptors. Based on characterization of their expression pattern in different tissues or expression during development or under particular physiological or patho-physiological conditions and based on the fact that the orphan receptors sequence-wise appear to belong to either established sub-families of 7TM receptors or together with other orphans in new families, it is believed that the majority of the orphan receptors are in fact important entities. As stated by representatives from the big pharmaceutical companies: Orphan 7TMs are "the next generation of drug targets" or "A neglected opportunity for pioneer drug discovery" (Wilson et al. Br.J.Pharmacol. (1998) 125: 1387-92; Stadel et al. Trends Pharmacol. Sci.; (1997) 18: 430-37; Howard A et al.; Trends Pharmacol. Sci. (2001) 22: 132-140). The problem is that it is very difficult to characterize orphan receptors and find their endogenous ligands, since no assays are available for these receptors due to the lack of specific ligands – a "catch 22" situation. The present invention provides a method of validating also the use of orphan receptors as drug targets through "pharmacological

knock-out" technology. By introducing metal ion binding sites in orphan receptors at locations where it is or will become known from work on multiple other 7TM receptors with known ligands and with binding and functional assays that binding of metal ions and metal ion chelates will act as either agonists or more common as antagonists, then it will be possible to make a potentially silent metal-ion site to be used *in vivo* as described for biological target molecules in general. It should be noted that due to the initial lack of knowledge of the endogenous ligand and therefore also lack of knowledge of the binding site for this ligand in the 7TM receptor, there is a certain risk that the introduced metal ion binding site can interfere with ligand binding or signal transduction. However, based on metal ion site engineering in multiple 7TM receptors and on mutational mapping of binding sites in multiple 7TM receptors, it will be possible to introduce such metal ion sites at different locations in the receptor in an attempt to eliminate this problem. Also each orphan receptor can be addressed with more than one metal-ion site.

Introduction and selection of a suitable metal ion site in a biological target molecule

It is generally known that metal-ion sites can be built into proteins by introduction of metal-ion chelating residues at appropriate sites. In a particularly preferred embodiment of the invention such sites are constructed at strategic sites in the biological target molecule with the purpose to serve as anchor sites for test compounds in a target validation process.

Mutagenesis - the nucleotide sequence encoding the target protein of interest may be subjected to site-directed mutagenesis in order to introduce the amino acid residue, which includes the metal-ion binding site or a residue, which is to serve as target for secondary site chemical interaction. Site-directed mutagenesis may be performed according to well-known techniques e.g. as described by Ho et al. Gene (1989) 77: 51-59. In a specific, non-limiting example the mutation is introduced into the coding sequence of the target molecule by the use of a set of overlapping oligonucleotide primer both of which encode the mutation of choice and through polymerisation using a high-fidelity DNA polymerase such as e.g. Pfu Polymerase (Stratagene) according to manufacturers specifications. The presence of the site-directed mutation event is subsequently confirmed through DNA sequence analysis throughout the genetic segment generated by PCR. In order to generate a metal-ion binding site this may involve the introduction of one or more amino acid residues capable of binding metal-ions including but not restricted to, for example His, Asp, Cys or Glu residues. In order to generate a suitable secondary site for chemical interactions any amino acid could be introduced. However, in a specific embodiment of

the invention a Cys residue is introduced as target for a bridging metal-ion site between the ligand and this residue or as a target for generation of a covalent bond.

Generally the mutated target molecule will initially be tested with respect to the ability to still constitute a functional, although altered, molecule through the use of an activity assay suitable in the specific case. It should be noted that although mutations in proteins may obviously occasionally alter the structure and affect the function of the protein, this is by far always the case. For example, only a very small fraction (less than ten) of the many hundred Cys mutations performed in rhodopsin as the basis for site directed spin-labelling experiments and in for example the dopamine and other 7TM receptors as the basis for Cys accessibility scanning experiments have impaired the function of these molecules. Similarly, in the bacterial transport protein Lac-permease almost all residues have been mutated and only a few of these substitutions directly affect the function of the protein. Notably Cys substitutions are generally well tolerated in membrane proteins. Mutations will often also be performed in the biological target molecule to confirm or probe for the chemical interaction of test compounds with other residues in the vicinity of the natural or the engineered metal-ion site as an often-integrated part of the general target validation method of the invention.

Metal-ion site engineering in protein targets in general - The method of the invention may suitably include a step of determining the location of, for example the metal ion binding amino acid residue(s) in a mutated protein and determining the location of at least one other amino acid residue in the vicinity of the metal ion binding amino acid residue, based on either the actual three-dimensional structure of the specific biological target molecule in question (e.g. by conventional X-ray crystallographic or NMR methods) or based on molecular models based on the primary structure of the specific molecule together with the three-dimensional structure of the class of molecules to which the specific molecule belongs (e.g. established by sequence homology searches in DNA or amino acid sequence databases).

In the biological target molecule, the metal-ion binding site may suitably be introduced to serve as an anchoring, primary binding site for the test compound, which can thereby be targeted to affect a site in the biological target molecule having one or more of the following properties (the metal-ion site may be placed either within or close to this site):

i) a site where the biological target molecule binds to another biological target molecule, for example a regulatory protein;

ii) a site which will control the activity of the biological target molecule in a positive or negative fashion (i.e. up-regulating or down regulating the activity of the biological target molecule), for example by an allosteric mechanism;

iii) a site where the binding of the test compound will directly or indirectly interfere with the binding of the substrate or natural ligand or the binding of an allosteric modulatory factor for the biological target molecule;

iv) a site where the binding of the test compound may interfere with the intra-molecular interaction of domains within the biological target molecule, for example the interaction of a regulatory domain with a catalytic domain;

v) a site where binding of the test compound will interfere with the folding of the biological target molecule, for example the folding of a protein into its active conformation;

vi) a site where the binding of the test compound will interfere with the cellular targeting of the biological target molecule;

vii) a site where the binding of the test compound will stabilize a conformation of the biological target molecule, which presents an epitope normally involved in protein-protein interactions in a non-functional form.

This list of properties is by no means exhaustive and only serves to give some examples of the possibilities which can be obtained by targeting the test compound to specific epitopes in the biological target molecule as part of its role as a tool in the target validation process of the present invention.

Metal-ion site engineering in 7TM proteins – Metal-ions play a fundamental role in biology - In natural proteins they are involved in functional purposes such as electron transfer or catalysis or in structural purposes stabilizing the three-dimensional structure of the protein. It is well known that also several integral membrane proteins include binding sites for metal ions. Regardless of whether the metal-ions play a functional or structural role, the specific properties exclusive to metals are utilized. In the proteins, the particular properties of the metal-ions may be fine-tuned by the amino acids defining the binding site to the application. An important general consideration is that metal-ions in fact offer the strongest binding interaction when viewed on a per atom basis compared to other ligands (I.D. Kuntz et al., Proc. Natl. Acad. Sci. USA, 96(18), 1999, pp. 9997-10002). The coordination of metal ions to metal ion binding sites is well characterized in numerous high-resolution X-ray and NMR structures of soluble proteins; for example, distances from the chelating atoms to the metal ion as well as the preferred conformation of the chelating side chains are known (e.g. J.P. Glusker, Adv. Protein Chem. 42, 1991, pp. 3-76; P. Chakrabarty, Protein Eng. 4, 1990, pp. 57-63; R. Jerigan et al., Curr. Opin. Struct. Biol. 4,

1994, pp. 256-263). Thus, metal-ion binding in proteins is one of the most well characterised forms of receptor-ligand-protein interactions known. Hence, characterising a metal ion-binding site in a membrane protein using, for example, molecular models and site directed mutagenesis can yield information about the structure of the membrane protein and importantly where the "ligand" (metal ion) binds (e.g. Elling et al. Fold. Des. 2(4), 1997, pp. S76-80).

The forces that control metal-ion binding - Amino acid residues that function as effective metal binding residues are typically those that contain electron-donation atoms (S, O or N) (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). Although this group includes amino acids such as Ser, Lys, Arg and Tyr the strongest interactions typically involve Asp, Glu, Cys and His. Binding of a metal-ion to a ligand (a residue or an organic compound) can be considered in terms of Lewis acid base theory (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). According to the Lewis concept, an acid is any species that can accept a pair of electrons, and a base is any species that can donate a pair of electrons. Consequently a metal acts as a Lewis acid when accepting an electron pair, and the ligand acts as a Lewis base when donating an electron pair. The nature of this electron transfer depends on the atoms involved, i.e. on the polarizability. On the basis of polarizability, metal ions may be classified as being hard/soft, hard meaning difficult to polarize. Important examples of cations such as Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and Ni^{2+} are classified as being 'borderline' (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76), i.e. neither hard nor soft. The softer the metal-ion is (more polarizability), the greater is the tendency to form π bonds between the metal-ion and the ligand, whereas a hard acid binds hard bases by ionic forces. Residues useful in metal-ion binding site engineering include for example aspartate, glutamate, histidine or cysteine. Aspartate and glutamate residues may carry one negative charge. Each oxygen atom has two lone pairs disposed at 120° to its C-O bond and in the plane of the carboxyl group. D.W. Christianson et al. (Am. Chem. Soc., 110(16), 1988, pp. 5560-5) showed that Zn(II) preferentially coordinates with the *syn* lonepair of the carboxylates (Z form) (Fig. 2). This was also observed by Carrell et al. (J. Am. Chem. Soc., 110(26), 1988, pp. 8651-6), which collected data from the Cambridge Structural Database. They showed that the syn-oriented lone pair of the carboxylate oxygen is preferred for cation binding. The metal-oxygen distance was in the range 2.3-2.6 Å and it is common to have 'direct bonding', in which the metal lies equidistant from the two oxygen atoms of the carboxylate group. In addition the metal-ion seems generally to lie in the plane of the carboxyl group. One carboxyl group can bind one or two metal ions, e.g. leucine aminopeptidase (Straeter et al., Biochemistry, 34(45), 1996, pp. pp. 14792-14800). Histidine residues are involved in binding of metal-ions in a

variety of enzymes and are the most abundant ligands in zinc binding sites (I. L. Alberts et al.,Protein Sci., 7(8), 1998, pp. 1700-1716). The imidazole ring exists in two tautomeric forms in which the proton is either on the Nε or Nδ nitrogen. The ratio is approximately 80% Nε-H and 20% Nδ-H (Fig. 3) (W.F. Reynolds et al., J. Am. Chem. Soc., 95(2), 1973, pp. 328-331). However, it is the Nδ-H form that predominantly binds the metal-ion (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63). Steric factors may be responsible for reversing the preference of the two tautomeres when the histidyl is bound to a metal ion. Another factor may be due to the structural properties of histidyl, where the Nε-metal coordinated form may be stabilized by Nδ-backbone carbonyl hydrogen bonding to a preceding turn in an α-helix (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63). Freeman (Inorganic Chemistry (G.L. Eichhorn, ed.), 1973, pp. 121-166) found that the metal-nitrogen bond of histidyl can be up to 30° from the imidazole plane and R. Candlen (J. Am. Chem. Soc., 110(26), 1967, pp. 8651-6) showed that the metal-Nε2-Cβ angles could range from 121° to 131° indicating a certain flexibility in the coordination, i.e. the metal-ion coordination does not have to be perfect for the metal to bind to the residue. The distance between the coordinated nitrogen and the metal is in the range 2.05-2.10 Å (I. L. Alberts et al.,Protein Sci., 7(8), 1998, pp. 1700-1716).

Coordination geometry - The most common structures of metal coordination spheres are octahedral, tetrahedral and square planar geometry's (F.A. Cotton et al, Basic Inorganic Chemistry (John Wiley & Sos, Inc.), 1995) (Fig. 4). The coordination number and geometry depend on the nature of the metal and the size of the ligands (metal ion chelators, protein sidechains, water etc.), and is usually as high as possible. The surrounding ligand atoms arrange in a geometry that minimizes the repulsive energy between them (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). Zinc(II) (Zn(II)) and copper(II) (Cu(II)) are examples of two metal ions useful in metal-ion binding site engineering. They are both well-investigated transition metals. Zinc is involved in many hydrolytic enzymes, such as carboxypeptidase and carbonic anhydrase, which utilize zinc in the active site (L. Stryer, Annu. Rev. Biochem., 37, 1968, pp. 25-50). Copper is also found in a number structures, e.g. azurin (E.T. Adam et al., J. Mol. Biol., 123(1), 1978, pp. 35-47). Zn(II) are most often found in a tetrahedral and octahedral geometry (D.W. Christianson, Adv. Protein Chem., 42, 1991, pp. 281-355). The mean zinc-ligand distance in protein coordination spheres is approximately 2 Å (2.05Å for histidine as a ligand) (P. Chakrabarti, Protein Eng., 4(1), 1990, pp. 57-63). Cu(II) are most often found in a square planar or a distorted octahedral geometry (see Fig. 4) (J.P. Glusker, Adv. Protein Chem., 42, 1991, pp. 1-76). The coordination octahedron of Cu^{2+} is found not to contain six bonds of equal lengths, but has four short bonds (~2.0Å) and two long bonds (~2.4Å) (in trans)

(Jahn and Teller, Proc. R. Soc. London, Ser. A 161, 1937, 220-235). Cu^{2+} usually bind its ligands (N & O) stronger than Zn^{2+} (Irving and Williams, Nature, 162, 1948, pp. 746) giving Cu^{2+} a different binding.

Engineering of new metal-ion binding sites - Due to the many favorable properties of metal-ions, engineering of new metal-ion binding sites in proteins have been performed in numerous cases although mostly in soluble proteins. Several examples where metal-ion site engineering has been included in the design strategy of *de novo* proteins have been reported. A common obstacle in *de novo* design of a protein is that the fold of the protein often resembles a state in between a molten globule and a native folded protein presumably due to the lack of specific tertiary interactions. Engineering of metal-ion sites have been utilized to introduce positive interactions in the protein fold stabilizing the folded native conformation as opposed to the molten globule state. A by now classic example of the elegant use of metal-ion site engineering was performed by Charlie Craik and coworkers in trypsin (J.N. Higaki, Biochemistry, 29, 1990, pp. 8582-8586). By introducing a histidine near the active site catalytic triad, a metal-ion switch was introduced. Upon metal-ion chelation, another histidine, that was normally part of the catalytic triad, would engage in metal-ion ligation with the introduced histidine and thereby render itself inaccessible for the normal catalytic function.

In one embodiment of the invention metal-ion sites are introduced in 7TM receptors. Engineering of artificial metal ion binding sites into membrane proteins has been employed to explore the structure and function of these proteins. Thus, C.E. Elling et al., *Nature 374*, 1995, pp. 74-77, have reported how the binding site for a proto-type antagonist for the tachykinin NK-1 receptor could be converted into a metal ion-binding site by systematic substitution of residues in the binding pocket with His residues. Accordingly, a tridentate zinc-site was constructed, composed of two histidine residues located in an *I* and *I+4* position at the exterior end of TM-V (V:01 and V:05) and a single His residue located in TM-VI (VI:24).

If side chains of amino acid residues participating in metal ion binding are known, it imposes a distance constraint on the protein structure which can be used in the interpretation of unknown protein structures (C.E. Elling and T.W. Schwartz, *EMBO J. 15*(22), 1996, pp. 6213-6219; C.E. Elling et al., *Fold. Des.* 2(4), 1997, pp. S76-80).

Recently the generation of an activating metal-ion binding site has been reported for the β_2-adrenergic receptor, where the binding site for the normal catecholamine ligands was

exchanged with a metal-ion site through specific substitutions in the binding pocket for the agonists (C.E. Elling et al, *PNAS 96*, 1999, pp. 12322-12327). This metal-ion binding site could be addressed also with metal-ions in complex with metal-ion chelators, i.e. small organic compounds binding metal-ions. The activating metal-ion site has successfully been transferred to another 7TM receptor, the tachykinin NK1 receptor (B. Holst et al., Mol. Pharmacol. 2000, 58: 263-270).

However, none of the above-mentioned documents address the concept of using a chemical "anchor" in the process of chemically optimising a compound for increased affinity and selectivity and optimising the test compound for suitable pharmacokinetic properties to be used in the target validation process *in vivo*.

The generally available metal-ion chelators or chelates will at best bind with affinities in the single digit micromolar range and therefore inhibit the function of the biological target molecule with similar or lower potency. Such low affinities and potencies will prohibit the use of the technology due to the fact that administration of metal-ions and metal-ion chelators in such concentrations will have multiple disturbing side-effects due to similar, non-specific, low affinity interaction of the test compounds with other – usually unknown - biological target molecules.

Metal-ion binding sites are constructed by mutating one or more amino acid residue in the biological target molecule into residues, which can bind metal-ions. These are usually His, Cys, Asp, or Glu residues but could also be Trp, Tyr, Ser, Thr, Lys, Arg, Asn, Gln and Met. Additionally an engineered site may utilize electron donating groups from the polypeptide backbone. It should be noted that also non-natural amino acids, which can bind metal-ions could be used, provided that a suitable method is employed to introduce these or a precursor for these - which can be chemically modified to become a metal-ion binding residue - into the biological target molecule. A metal-ion site includes one, two, three or four amino acid residues although more residues also can occur. In a specific embodiment of the invention the metal-ion binding site includes two residues, which allow for the metal-ion to bind also the metal-ion chelator. Often single metal-ion binding residue(s) are already present in the biological target molecule in a suitable location and consequently only a single extra metal-ion binding residue needs to be introduced in the spatial vicinity through mutational substitution of the residue found in the wild-type of the biological target molecule.

In order for two or more residues to make up a metal-ion binding site, the metal-ion binding atom of the amino acid residues need to be located or be able to move into a location which satisfies the geometrical criteria for making a metal-ion binding site with the particular metal-ion used, which will be known to a person skilled in the art (I. Lalbert, 1998, Protein Science, 7: 1700-1716, B. Lvallee, 1990, Biochemistry, 29(24):5647-5659), Hellinga HW et al., J. Mol. Biol., 222(3), 1991, pp. 763-85). When engineering a metal-ion binding site in a 7TM receptor a general classification of the site may be performed based on the general placement of the introduced amino acid sidechains chelating the metal-ion. The metal-ion binding sites may be described as being either intra-helical, i.e. the residues composing the site are located on the same transmembrane helix; as being inter-helical, i.e. the residues composing the site are located on at least two transmembrane helices; or generally as involving residues within transmembrane segments and/or loops and turns of the protein. Whereas the residues defining an intra-helical metal-ion binding site are located on the same face of the helix, for example in an *i, i+4* manner, residues defining inter-helical residues are located on opposing faces of the involved helices and the site may be composed of one or more residues from each helix.

In a particularly preferred embodiment of the invention one or more of the employed metal-ion binding residues will be a sulphur containing residue such as Cys which binds for example Ru(II), Pd(II) and Pt(II) particularly well.

An important part of the present invention is to increase the affinity of the test compound through the establishment of just a single or a few secondary chemical interaction(s) besides the anchoring binding of the metal-ion part of the complex. Thus, in order to facilitate the establishment of such a suitable secondary chemical interaction, the anchoring metal-ion sites are in a preferred embodiment of the invention built into the biological target molecules at sites where knowledge from the known three-dimensional structure or from models of the three-dimensional structure indicate, that a suitable chemical moiety is present in the vicinity of the engineered metal-ion site. For example, if an acid residue, Asp or Glu, or a basic residue, Arg, Lys or His, are found in a suspected exposed manner and in a supposed charged form in an certain epitope of a biological target molecule, a metal-ion site can be build in the vicinity of the charged residue, and chemical modifications of a suitable metal-ion chelate can be performed in order to establish a charge-charge interaction with the supposedly charged residue in the biological target molecule. In a similar manner, sites for engineering of metal-ion sites can be chosen in order to establish other types of suitable, secondary site chemical interactions through appropriate chemical modifications of the test compound. In a preferred embodiment of the invention, the secondary chemical interaction includes the

binding of second metal-ion between the test compound and the secondary site residue. In a particular embodiment, the secondary chemical interaction is a covalent bond, for example established with a thiol-containing residue, for example a Cys, or an amine-containing residue, for example a Lys. It should be noted that chemically reactive groups with suitable chemical reactivity can be introduced into the test compound in order for these to selectively react with the intended, particular secondary site residue in the biological target molecule - for example a Cys - and not with such residues in general in the biological target molecule or in the test animal in general, due to the close proximity in which the reactive group on the test compound is brought through the binding of the test compound to the metal-ion site in the biological target molecule.

In a specific embodiment of the invention not only a metal-ion binding site is introduced into the biological target molecule, but also one or more residue(s), which can form a suitable secondary site chemical interaction - as indicated above - are introduced. Also in this case, it is required that this extra substitution is made as a relatively silent substitution fulfilling the criteria, which were discussed in relation to the silent metal-ion binding site. Any type of natural or non-natural amino acid residue could be introduced with the purpose of establishing secondary site interactions. However, in a preferred embodiment of the invention, the residue introduced for the establishment of a secondary site interaction is a Cys residue due to the fact, that this residue can be introduced in a relatively silent manner at multiple sites in biological target molecules such as for example membrane proteins such as 7TM receptors and transporters. Moreover, a Cys residue is particularly suited for establishing secondary site interactions such as a metal-ion bridge or a covalent bond.

Selection of a suitable metal-ion binding site in 7TMs

As discussed above, metal-ion binding sites in 7TMs may be found in the helices or in the loops. The binding site may be intrehelical, i.e. within the same helix or interhelical, i.e. involving two or more helices. In the following is given single positions in the different transmembrane segments of a 7TM; these single positions are positions which are suitable metal-ion binding sites:

Single Positions:

transmembrane segment-I:

[I:01]

[I:03]

[I:04]
[I:06]
[I:07]
[I:10]
[I:11]
[I:14]
[I:17]
[I:18]
[I:21]
[I:22]

or in positions

transmembrane segment-II:
[II:05]
[II:06]
[II:07]
[II:09]
[II:10]
[II:13]
[II:14]
[II:17]
[II:18]
[II:20]
[II:21]
[II:22]
[II:24]
[II:25]
[II:26]

or in positions

transmembrane segment-III:
[III:04]
[III:05]
[III:08]
[III:09]
[III:11]
[III:12]
[III:13]

[III:15]
[III:16]
[III:17]
[III:18]
[III:19]
[III:20]
[III:21]
[III:22]
[III:23]
[III:24]
[III:25]
[III:26]
[III:27]
[III:28]
[III:29]
[III:30]
[III:32]

or in positions

transmembrane segment-IV:
[IV:01]
[IV:02]
[IV:05]
[IV:06]
[IV:09]
[IV:10]
[IV:12]
[IV:13]
[IV:14]
[IV:16]
[IV:17]
[IV:18]
[IV:19]
[IV:20]

or in positions

transmembrane segment-V:
[V:01]
[V:04]

[V:05]
[V:08]
[V:09]
[V:12]
[V:13]
[V:16]
[V:17]
[V:20]
[V:21]
[V:23]
[V:24]
[V:27]
[V:28]

or in positions

transmembrane segment-VI:

[VI:-06]
[VI:-04]
[VI:-03]
[VI:-02]
[VI:-01]
[VI:01]
[VI:02]
[VI:03]
[VI:05]
[VI:06]
[VI:08]
[VI:09]
[VI:12]
[VI:13]
[VI:16]
[VI:17]
[VI:19]
[VI:20]
[VI:21]
[VI:23]
[VI:24]

or in positions

transmembrane segment-VII:

[VII:02]
[VII:03]
[VII:05]
[VII:06]
[VII:07]
[VII:08]
[VII:09]
[VII:10]
[VII:11]
[VII:12]
[VII:13]
[VII:15]
[VII:16]
[VII:17]
[VII:19]
[VII:20]

In the following is given preferred single positions in the different transmembrane segments of a 7TM; these single positions are preferred positions which are suitable metal-ion binding sites:

Preferably - Single

TM-I:

[I:03]
[I:07]
[I:10]
[I:14]

TM-II:

[II:17]
[II:18]
[II:20]
[II:21]
[II:24]
[II:25]

TM-III:

[III:04]

[III:05]

[III:08]

[III:09]

[III:19]

[III:23]

[III:27]

[III:29]

[III:32]

TM-IV:

[IV:12]

[IV:13]

[IV:16]

[IV:17]

[IV:19]

[IV:20]

TM-V:

[V:01]

[V:04]

[V:05]

[V:08]

[V:09]

[V:12]

TM-VI:

[VI:-01]

[VI:-02]

[VI:09]

[VI:16]

[VI:19]

[VI:20]

[VI:23]

[VI:24]

TM-VII:

[VII:02]

[VII:03]

[VII:06]

[VII:07]

[VII:10]

[VII:16]

In the following is given specific sites in the different transmembrane segments of a 7TM; these specific sites are sites which are suitable metal-ion binding sites:

transmembrane segment-I:

[I:03;I:07]

[I:06;I:10]

[I:10;I:14]

[I:14;I:18]

[I:17;I:21]

[I:18;I:22]

or in positions

transmembrane segment-II:

[II:05;II:09]

[II:06;II:10]

[II:09;II:13]

[II:17;II:21]

[II:20;II:24]

[II:21;II:25]

or in positions

transmembrane segment-III:

[III:04;III:08]

[III:05;III:09]

[III:08;III:12]

[III:09;III:13]

[III:11;III:15]

[III:12;III:16]

[III:15;III:19]

[III:19;III:23]
[III:20;III:24]
[III:21;III:25]
[III:22;III:26]
[III:23;III:27]
[III:24;III:28]

or in positions

transmembrane segment-IV:
[IV:01;IV:05]
[IV:02;IV:06]
[IV:05;IV:09]
[IV:06;IV:10]
[IV:12;IV:16]
[IV:13;IV:17]
[IV:14;IV:18]

or in positions

transmembrane segment-V:
[V:01;V:05]
[V:04;V:08]
[V:05;V:09]
[V:08;V:12]
[V:09;V:13]
[V:12;V:16]
[V:13;V:17]
[V:16;V:20]
[V:17;V:21]
[V:20;V:24]
[V:23;V:27]
[V:24;V:28]

or in positions

transmembrane segment-VI:
[VI:-04;VI:01]
[VI:-03;VI:02]
[VI:-02;VI:03]
[VI:01;VI:05]
[VI:02;VI:06]
[VI:05;VI:09]

[VI:08;VI:12]
[VI:09;VI:13]
[VI:13;VI:17]
[VI:16;VI:20]
[VI:17;VI:21]
[VI:19;VI:23]
[VI:20;VI:24]

or in positions

transmembrane segment-VII:
[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]
[VII:07;VII:11]
[VII:09;VII:13]
[VII:12;VII:16]
[VII:13;VII:17]
[VII:15;VII:19]
[VII:16;VII:20]

or in positions

transmembrane segment-I and -II
[I:01;II:26]
[I:01;II:25]
[I:01;II:22]
[I:03;II:25]
[I:04;II:26]
[I:04;II:25]
[I:04;II:22]
[I:07;II:25]
[I:07;II:22]
[I:07;II:21]
[I:07;II:18]
[I:10;II:18]
[I:10;II:14]
[I:10;II:13]
[I:11;II:18]
[I:11;II:14]
[I:14;II:18]

[I:14;II:13]
[I:14;II:14]
[I:14;II:10]
[I:18;II:10]
[I:18;II:07]

or in positions

transmembrane segment-I and -VII
[I:03;VII:07]
[I:03;VII:03]
[I:07;VII:07]

or in positions

transmembrane segment-II and -III
[II:20;III:04]
[II:24;III:04]

or in positions

transmembrane segment-II and -VII
[II:17;VII:10]
[II:18;VII:10]
[II:21;VII:07]

or in positions

transmembrane segment-III and -IV
[III:05;IV:17]
[III:05;IV:20]
[III:09;IV:16]
[III:09;IV:17]

or in positions

transmembrane segment-III and –V
[III:05;V:01]
[III:05;V:04]
[III:05;V:08]
[III:09;V:01]
[III:09;V:04]
[III:09;V:08]
[III:12;V:08]
[III:13;V:08]
[III:13;V:12]

[III:16;V:12]
[III:16;V:16]
[III:17;V:16]
[III:19;V:16]
[III:20;V:16]
[III:23;V:27]
[III:23;V:23]
[III:26;V:20]
[III:26;V:24]
[III:26;V:27]
[III:27;V:23]
[III:27;V:27]
[III:30;V:23]
[III:30;V:27]

or in positions

transmembrane segment-III and -VI
[III:05;VI:16]
[III:08;VI:16]
[III:08;VI:13]
[III:09;VI:13]
[III:12;VI:13]
[III:13;VI:09]
[III:16;VI:09]
[III:19;VI:09]
[III:19:VI:06]
[III:19;VI:05]
[III:19;VI:09]
[III:22;VI:05]
[III:23;VI:09]
[III:25;VI:02]
[III:25;VI:-03]
[III:26;VI:02]
[III:26;VI:-03]
[III:26;VI:-02]
[III:26;VI:-06]
[III:27;VI:-01]

[III:27;VI:-02]
[III:29;VI:-02]
[III:30;VI:-06]
[III:30;VI:-02]
[III:32;VI:-02]

or in positions

transmembrane segment-III and -VII
[III:04;VII:07]
[III:05;VII:06]
[III:09;VII:09]
[III:08;VII:06]
[III:08;VII:10]
[III:11;VII:10]
[III:12;VII:06]
[III:12;VII:10]
[III:12;VII:12]
[III:15;VII:16]
[III:19;VII:16]

or in positions

transmembrane segment-IV and -V
[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

or in positions

transmembrane segment-V and -VI
[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]
[V:09;VI:20]
[V:09;VI:21]
[V:09;VI:17]

or in positions

transmembrane segment-VI and -VII

[VI:23;VII:02]
[VI:23;VII:05]
[VI:20;VII:02]
[VI:19;VII:05]
[VI:19;VII:02]
[VI:16;VII:02]
[VI:16;VII:06]
[VI:16;VII:09]
[VI:16;VII:10]
[VI:12;VII:08]
[VI:12;VII:09]
[VI:09;VII:12]
[VI:09;VII:15]
[VI:05;VII:16]
[VI:05;VII:15]

In the following is given preferred specific sites in the different transmembrane segments of a 7TM; these specific sites are preferred sites, which are suitable metal-ion binding sites:

Preferably - *Intra*helical

TM-I:
[I:03;I:07]
[I:10;I:14]

TM-II:
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

TM-III:
[III:04;III:08]
[III:05;III:09]

TM-IV:
[IV:12;IV:16]

[IV:13;IV:17]

TM-V:

[V:01;V:05]

[V:04;V:08]

[V:05;V:09]

TM-VI:

[VI:16;VI:20]

[VI:19;VI:23]

[VI:20;VI:24]

TM-VII:

[VII:02;VII:06]

[VII:03;VII:07]

[VII:06;VII:10]

Preferably - *Inter*helical Sites

TM-I / TM-VII

[I:03;VII:07]

[I:07;VII:07]

[I:03;VII:03]

TM-II / TM-III

[II:20;III:04]

[II:24;III:04]

TM-II / TM-VII

[II:17;VII:10]

[II:18;VII:10]

[II:21;VII:07]

TM-III / TM-IV

[III:05;IV:17]

[III:05;IV:20]

[III:09;IV:16]
[III:09;IV:17]

TM-III / TM-V

[III:05;V:01]

TM-III / TM-VI

[III:08;VI:16]
[III:19;VI:09]
[III:23;VI:09]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:32;VI:-02]

TM-III / TM-VII

[III:08;VII:06]
[III:19;VII:16]

TM-IV / TM-V

[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

TM-V / TM-VI

[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]

TM-VI / TM-VII

[VI:23;VII:02]
[VI:23;VII:06]
[VI:19;VII:02]
[VI:19;VII:06

Selection of a suitable test compound (metal-ion chelator or metal-ion chelate) for use in the target validation process

A very important step in the target validation process suitable for the use described herein is a selection of a suitable metal-ion chelate for administration to the test animals (and to the control animals). Instead of applying a metal-ion chelate, a metal-ion chelator may be employed and in such cases the chelator may be administered together with a suitable metal ion (e.g. in the form of a suitable salt, complex etc) or the metal-ion chelate may be formed *in situ* after administration by means of metal ions present in the body. The metal ion chelate (or a transport or depot form thereof) has ideally the following properties:

1. it can easily be administered to the test animal
2. it can easily reach the site of action within the test animal
3. it has a suitable binding affinity to the silent metal ion binding site in the biological target molecule within the test animal
4. it functions as a switch, i.e. the function of the biological target molecule within the test animal is either turned on or off
5. it remains on the site of action in a suitable concentration for a suitable time period being sufficient to determine the relevant biochemical, physiological or behavioural effect
6. it is relatively safe for the test animal and does not have undesirable effects on relevant biochemical, physiological or behavioural parameters in the wild-type, i.e. non genetically modified animal

In the following a description of suitable test compounds is given followed by a discussion of parameters which are relevant in connection with a structure based selection of the test compounds (e.g. items 3, 4, 5 and 6 above) and, finally is given a discussion of parameters which are relevant in connection with a pharmacokinetic and/or biopharmaceutical selection of the test compounds (items 1, 2 and 5 above).

Chemical compounds, which are suitable for use in target validation processes involving biological target molecules having a metal-ion site are any compound that is capable of forming a complex with a metal ion.

In general, complexes of interest are chelates comprising three major parts: the functionalised chelator, the metal ion *(central metal or coordinated metal)*, and displaceable ligands bound to the metal. When the metal chelate binds to the biological

target the displaceable ligands are exchanged with groups from the biological target or water.

Functional groups in a ligand attached to the metal ion are the ligand's coordinating groups. A ligand attached directly through only one coordinating atom (or using only one coordination site on the metal) is called a monodentate ligand. A ligand that may be attached through more than one atom is multidentate, the number of actual coordinating sites being indicated by the terms bidentate, tridentate, tetradentate and so forth. Multidentate ligands attached to a central metal by more than one coordinating atom are called chelating ligands, or chelators. A chemical compound for use in the present context is at least bidentate, i.e. it is a so-called metal-ion chelator.

More specifically, a chemical compound for use in a library according to the invention has at least two heteroatoms, similar or different, selected from the group consisting of nitrogen (N), oxygen (O), sulphur (S) and phosphorous (P).

In the choice of suitable displaceable ligands it is very important to find the right balance between too tight binding to the metal-ion and too weak binding to achieve a useful complex. An example is the fact that chloride binds tightly to the metal-ion, whereas dimethylsulfoxide has a much weaker interaction. The interaction ability of a displaceable ligand is usually correlated with the acidity of the corresponding acid, and a very useful leaving group is triflate, which is the corresponding base of the highly acidic trifluorometyl sulphonic acid.

The ability of a metal-ion chelator complex to engage in reactions that result in replacing the displaceable ligands in the coordination sphere, e.g. replacing the chelator compound with an extracellular protein, is described by its lability. Those complexes for which such substitution reactions are fast are called *labile*, whereas those for which such substitution reactions proceed slowly (or not at all) are called *inert*. These terms relates to the kinetic parameter, the *rate constants* (k), of the reaction. A practical definition of inert complexes, are those whose substitution reactions have a half-live ($T_{1/2}$) longer than one minute.

It is of importance that the metal-ion chelator complex is able to reach and specifically bind to the modified receptor in the genetically modified animal as an intact complex. However, in the body fluids there are several metal-ion binding molecules to compete with the chelator for the metal ion. In the extracellular fluid different metal-ion binding proteins

such as albumin and α-macroglobulin are very abundant. They are binding the metal ion with an affinity in the micromolar range, but with a considerable capacity.

In a preferred embodiment of the present invention inert complexes, which do not freely exchange the metal-ion with other metal-ion binding molecules in the body fluid, and which have a high affinity and selectivity for the genetically modified receptor, are used. The inertness of metal ion in a complex, is increased with the number of the period (in the periodic table), e.g. complexes with group 8, iron (Fe) has a T½ measured in seconds, ruthenium (Ru) has a T½ measured in hours and osmium (Os) is almost unable to engage in complex reactions. A similar pattern is observed for the other members of the transition metals. Thus, in a preferred embodiment of the invention, transition metals in period 5 of the periodic table are used as the preferred metal-ions in the test compounds. Although metal-ions in period 4 have a faster exchange rate, such metal-ions can also be employed.

In order for the test compounds to be useful in the *in vivo* setting of the genetically modified animal, the affinity and selectivity of the metal-ion chelate for the metal-ion site in the engineered biological target molecule should have a reasonable high affinity and therefore it often needs to be improved. This is achieved through chemical optimisation for secondary site interaction(s). Such a chemical optimisation can be performed either in a random fashion or in a more targeted fashion, utilising structural information on the target protein. Typically, the optimisation is based on a collection of test compounds, i.e. based on selected libraries of test compounds.

The present invention aims at providing chemical compounds and collections of chemical compounds (libraries), which are suitable for use in optimising the primary and secondary interactions with a biological target molecule (i.e. optimising the interaction between the metal-ion chelate and the metal-ion binding site in the biological target molecule and, furthermore, optimising the secondary interactions of the metal-ion chelate with suitable functional groups in the vicinity of the metal-ion binding site).

A library according to the invention contains normally three or more chemical compounds. Typically a library based on structural information from biological target molecules contains at least 3 and often at the most 100 compounds.

Optimising the binding to the metal-ion binding site of the biological target molecule containing a metal-ion binding site

Primary interaction, the metal complex

The nature of the complex can be altered depending on the metal-ion binding site in the biological target molecule. It is usually advantageous in the described target validation process to have inert complexes. Preferably, complexes capable of forming an irreversible binding with the biological targets should be made. Furthermore, the chelator with its metal should be compatible with the route of administration and time course of the experiment.

The types of ligands can roughly be divided into three groups; (i) the pure σ-donating ligands, e.g. amines or thiols, (ii) ligands with additional π-back bonding to an electron deficient π systems, e.g. pyridines, and (iii) ligands with additional π-back bonding to an electron rich π systems, e.g. thiophenes.

To enforce a low electron density at the metal centre, use of ligands with back bonding capabilities is favorable, and this is further improved with electron deficient π systems. Firstly, this strengthens the binding between the chelator and the metal, and secondly, removing electron density from the metal allows the metal site in the target to form stronger interactions with the metal. Another aspect to be considered is the size of the metal centre. If the metal is among the heavier metals, a larger heteroatom may be of value, e.g. sulphur atoms instead of nitrogen atoms. Based on these considerations, chelators containing e.g. pyridine moieties are of interest, as well as crown ether types like tetraazacyclononane or cyclam. Possible chelators advantageous for the task are 2,2'-bipyridine, 8-hydroxyquinoline, 8-mercaptoquinoline, and 2-(2-pyridyl)thiophenol.

Secondly, there is the choice of metal. Zinc and copper have proven useful for complexing to bipyridyls, but both are forming labile complexes, where interchange of ligands is fast. They may be useful for the purpose in certain cases, but metals forming more stabile complexes are preferable. Metals represented by technetium, ruthenium, rhodium, palladium, osmium, platinum etc. are known to form more inert complexes. The various metals have different electronic properties, thus different metals will show different preferences for various electronic environments, both in the metal site in the target, as well as from the chelator.

Also the oxidation level is important for complexation reactions. Thus in a preferred embodiment of the invention specific oxidation states of metal ions are used which create the most stable complexes, for example Ru(II), Rh(III), Pd(II), Pt (II).

Concerning the coordinating groups in the chelator, "soft" atoms (those with a low electronegativity) make more inert complexes with the metal-ions compared to "hard" atoms.

Finally, the interaction between the metal and the metal site in the protein target can be modulated. Both histidine, with its imidazole moiety, and cysteine, with its thiol group, have proven successful as ligands to the metal. Cysteine is a pure σ-donor, while histidine also includes additional π-back bonding capabilities. Other residues that may be used in the metal sites are glutamates and aspartates. As for the interactions between the chelator and the metal, the soft metal ions e.g. Pd(II), Ru(II), Rh(III) and Pt(II) have a high tendency to form inert interaction with the metal-ion binding site modified biological target molecule, dependent on which residues that form the metal ion site.

Optimising for secondary interactions

Based on a selected or randomly chosen metal-ion chelate or metal-ion chelator, other basic bi-functional metal-ion chelates or metal-ion chelators or libraries are constructed in which the compounds have both an anchoring *metal-ion binding moiety*, which conveys the ability to bind to the metal-ion binding site in the biological target molecule, as well as a *variable moiety*, which is varied chemically to probe for improved interactions with specific parts of the biological target molecule located spatially adjacent to the metal-ion binding site. In one embodiment of the target validation process screening of chemical moieties or libraries is performed in the variable part of the test compound. In a preferred embodiment of the invention libraries of test compounds are constructed based on structural knowledge of the biological target molecule. Thus, the test compounds for use according to the invention may be chemically optimised by introducing functional groups, which are able to establish an interaction with specific chemical groups located in the vicinity of the metal-ion site in the biological target molecule.

Secondary interactions, increasing the affinity

When the metal complex bridging the biological target molecule and the chelator has been established, a secondary interaction between the chelator and residues in the protein

target can be introduced. These are obtained by functionalising the chelators with proper functional groups at proper distances from the metal centre.

The bonds formed may be of, but not limited to, one or several of the following types:

covalent bonds, ionic interactions, ion-dipole interactions, dipole-dipole interactions, hydrogen bond interactions, hydrophobic interactions, pi-stacking interactions, edge-on aromatic interactions, dispersion and inductions forces or metal complex interactions. More specifically, stronger bonds and interactions are of primary interest for the purpose of the invention, i.e.

1. irreversible or slowly reversible covalent bonds.
2. charge-charge interaction (introduction of charged groups such as ammonium, phosphonium or sulphonium groups, or ionisable groups such as amino or carboxy groups).

Additional interactions might contribute to the effect, i.e.

3. hydrogen bond interactions (introduction of e.g. hydroxy, amino, keto amide, sulphonyl or other groups capable of interacting via formation of a hydrogen bond).
4. cation-π interactions (introduction of cationic groups such as ammonium groups or introduction of an aromatic moiety)
5. aromatic-aromatic interactions (introduction of an aromatic group in the test compound).
6. hydrophobic interactions (introduction of hydrophobic groups).
7. a second metal-ion bridge or anchor established through a metal-ion binding moiety on the variable part of the compound and a metal-ion binding residue on the biological target molecule.

The functionality can be introduced in a targeted manner, targeting either a natural residue in the protein target, or targeting an engineered residue, optimised for the interaction.

Further functional groups may be introduced to increase affinity, selectivity, or physicochemical properties.

The reactive groups used for forming secondary interactions should be compatible with the route of administration and time course of the experiment. The listed groups provide a proper range of reactivity that will be used for the specific biological target proteins and experiments.

Optimisation of absorption, distribution, metabolism, and excretion of the test compound

The aim of the above-mentioned manipulation or chemical modification of the test compounds is to improve the properties of a test compound with respect to:

1. its binding affinity to the silent metal ion binding site in the biological target molecule within the test animal,
2. its function as a switch, i.e. its ability to turn an effect on or off *in vivo*
3. its ability to remain at the site of action for a sufficient period of time
4. its suitability as a test compound in animals

The properties of a test compound may also be changed with respect to its pharmacokinetic and/or biopharmaceutical properties cf. the discussion below.

The test compound, which has been optimised for binding affinity and selectivity for the biological target molecule will often not have the appropriate pharmacokinetic etc. properties to be useful in the *in vivo* setting of the genetically modified animal. However, since the test compound is only to be used in animal experiments and not directly in human beings, there is a rather large degree of freedom in respect of what can be done to improve, for example, the pharmacokinetic properties of the test compound as compared to what can be done with a compound which is going to be used for the treatment of human patients.

Thus in a specific embodiment of the invention, the test compound, which has been optimised for binding affinity and selectivity for the biological target molecule is further optimised for appropriate properties of absorption, distribution, metabolism, and excretion either through further organic chemical modifications or through association of the test-compound with a molecule providing the desired pharmacokinetic properties.

A change of the pharmacokinetic properties of a test compound may include a change in the absorption rate, the plasma half-life, the distribution, the metabolism and/or the elimination of the test compound.

A change in the biopharmaceutical properties of a test compound may include a change in the water-solubility (e.g. by salt or complex formation), in the lipid solubility (e.g. by formation of a salt or a complex) and/or in the particle size of the test compound. The formulation technique chosen depends on which properties of the test compound. that are desirable. Whether a test compound is presented in dissolved form or not depends on the available administration route. Thus, if the test compound is administered orally, it can be presented in dissolved or non-dissolved form. However, it is contemplated that the test compound must dissolve before it can be absorbed and enter the systemic circulation.

Test compounds may by themselves have an undesirable plasma half-life. In order, for example, to increase the plasma half-life of a test compound, the test compound may be chemically modified in such a manner that it is linked to a larger molecule (carrier) optionally via a spacer. Such techniques are well-known to a person skilled in the art and suitable carriers are e.g. macromolecular carriers of natural, synthetic or semisynthetic origin like e.g. polysaccharides (e.g. dextrans, cellulose or cellulose derivatives), polypeptides, proteins, polyethylene glycols, poly lactic acids, poly glycolic acids, poly (lactic-glycolic) acids, acrylic acid polymers, ethyl vinyl acetate polymers, hyaluronic acids, gelatines. The test compound may either through a covalent or a non-covalent bond be directly linked to the macromolecular carrier or it may be indirectly linked to the macromolecular carrier via a spacer. Such a spacer may serve the following purposes:

1. The spacer includes chemically functional groups, which make it possible to react at the one end with the carrier and at the other end with the test compound. Thus, the spacer is necessary in order to connect the carrier and the test substance.
2. The spacer enables a distance between the carrier and the test substance so that a possible interaction from the carrier on the biological target molecule is eliminated or significantly reduced.

A suitable spacer may be a short chain peptide, a poly- or oligoethyleneglycol, or a short chain polysaccharide in which one or more hydroxy groups optionally have been substituted with e.g. amino, sulphate, amide, ester or ether groups.

A test compound may also be modified in order to improve the localization of the test compound after administration to a test animal. Thus, in a specific embodiment of the invention incorporation of specific chemical groups or association of the test compound with certain specific molecules, peptides, proteins or other macromolecules provide the test compound with the property of accumulating or localizing to specific sites within the body, e.g. to the brain (by passage of the blood brain barrier), to tumor-associated tissue or the like. The test compound can also be modified to pass through biological membranes and thereby become accessible to intracellular biological target molecules.

The distribution of the test compound may be changed by incorporating carriers, which mainly are hydrophilic or lipophilic of nature. In these cases, the carriers may be an integral part of the test compound. Moreover, pharmaceutically acceptable excipients normally used in localised drug delivery may be included in the dosage form.

Other methods for sustaining the effect of the test compound (or a transport form thereof) can be employed. Thus, controlled release formulation techniques well-known for at person skilled in the art of pharmaceutical formulation can be employed, e.g. in order to prepare a controlled release composition containing the test compound and from which the test compound is only slowly released over a time period of, e.g., 2-20 hours or even longer.

Controlled release formulation techniques are known for many kinds of dosage forms including oral, topical, parenteral, rectal, ocular and nasal dosage forms.

Chemical compounds (test compounds)

Besides the chemical structure, the test compounds normally fulfil certain criteria with respect to molecular weight (at the most 3000 such as, e.g., at the most 2000, at the most 1500, at the most 1000, at the most 750, at the most 500), number of hydrogen bond donors (at the most 15 such as, e.g. at the most 13, 12, 11, 10, 8, 7, 6 or at the most 5) and number of hydrogen bond acceptors (at the most 15 such as, e.g. at the most 13, 12, 11, 10, 8, 7, 6 or at the most 5). However, there may be cases where the molecular weight, number of hydrogen bond donors and/or number of hydrogen bond acceptors of a test compound of a library of the invention have other values than the above-mentioned.

Chemical compounds, which are suitable for use in target validation processes involving biological target molecules having a metal-ion site, are any compounds that are capable of forming a complex with a metal ion.

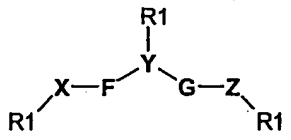
More specifically, a chemical compound for use according to the invention has at least two heteroatoms, similar or different, selected from the group consisting of nitrogen (N), oxygen (O), sulphur (S), selenium (Se) and phosphorous (P).

Suitable metal ion chelators may have any log K value. Normally a log K value from about 1 to about 50 is suitable such as, e.g. a log K value in a range of from about 3 to about 40, such as, e.g., from about 3 to about 30, from about 3 to about 26, from about 3 to about 18, from about 3 to about 15, from about 3 to about 12, from about 4 to about 10, from about 4 to about 8. In certain cases, the log K value may be from about 4.5 to about 7, from about 5 to about 6.5 such as from about 5.5 to about 6.5. K is an individual complex constant (also denoted equilibrium or stability constant). The constant's subscript 1, 2, 3 etc. indicates which coordination step the constant is valid for, i.e. K_1 is the complex constant for the coordination of the first ligand, K_2 is for the second ligand and so forth. log K can be determined as described in W.A.E. McBryde, "A Critical Review of Equilibrium Data for Protons and Metal Complexes of 1,10-Phenanthroline, 2,2'-bipyridyl and related Compounds." Pergamon Press, Oxford, 1978.

Test compounds, which have been found to be useful in the present methods, are typically compounds comprising a heteroalkyl, heteroalkenyl, heteroalkynyl moiety or a heterocyclyl moiety for chelating the metal ion. The term "heteroalkyl" is understood to indicate a branched or straight-chain chemical entity of 1-15 carbon atoms containing at least one heteroatom. The term "heteroalkenyl" is intended to indicate a branched or straight-chain chemical entity of 2-15 carbon atoms containing at least one double bond and at least one heteroatom. The term "heteroalkynyl" is intended to indicate a branched or straight-chain chemical entity of 2-15 carbon atoms containing at least one triple bond and at least one heteroatom. The term "heterocyclyl" is intended to indicate a cyclic unsaturated (heteroalkenyl), aromatic ("heteroaryl") or saturated ("heterocycloalkyl") group comprising at least one heteroatom. Preferred "heterocyclyl" groups comprise 5- or 6-membered rings with 1-4 heteroatoms or fused 5- or 6-membered rings comprising 1-4 heteroatoms. The heteroatom is typically N, O, S, Se or P, normally N, O or S. The heteroatom is either an integrated part of the cyclic, branched or straight-chain chemical entity or it may be present as a substituent on the chemical entity such as, e.g., a thiophenol, phenol, hydroxyl, thiol, amine, carboxy, etc. Examples of heteroaryl groups

are indolyl, dihydroindolyl, furanyl, benzofuranyl, pyridinyl, pyrimidinyl, quinolinyl, triazolyl, imidazolyl, thiazolyl, tetrazolyl and benzimidazolyl. The heterocycloalkyl group generally includes 2-20 such as 3-20 carbon atoms, and 1-4 heteroatoms.

Particularly useful chemical compounds of the present invention are those having at least two heteroatoms connected according to the general formula I abbreviated as Che-R1



Formula I

wherein F is N, O, S, Se or P; and G is N, O, S, Se or P;

X, Y and Z, which are the same or different, are straight or branched C_1-C_{12} alkyl, C_1-C_{12} alkenyl, C_1-C_{12} alkynyl, C_1-C_{12} cyclyl, aryl, C_1-C_{12} heteroalkyl, C_1-C_{12} heteroalkenyl, C_1-C_{12} heteroalkynyl, C_1-C_{12} heterocyclyl, heteroaryl;

R^1 may be present anywhere on the X, Y and/or Z moiety and it may be present on X, Y and/or Z up to as many times as possible, i.e. if X is $-CH_2-CH_2-$, then R^1 may be present on the first and/or second carbon atom one or several times; R^1 could optionally be hydrogen;

X may together with Y and/or Z fuse to form a cyclic ring system;
Y may together with X and/or Z fuse to form a cyclic ring system;
X, Y and Z may together fuse to form a cyclic ring system;

R^1 corresponds to a structure –A-B-C, wherein the element A is a coupling or connecting moiety, B is a spacer moiety and C is a functional group; –B- may be substituted one or more times with a further C, which may be the same or different, and

A linked to be –A-B-C is selected from the group consisting of:

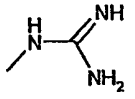
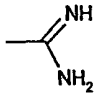
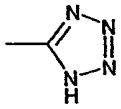
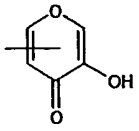
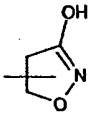
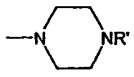
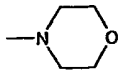
-O-, -S-, -NH-, -N=, -N<, -CH_2-, -C(=O)-, -PO_3-, -PO_2NH-, -$NHPO_2$, -NHP(O)<, -C≡C-, -CH=CH-, -SO-, -SO_2-, -COO-, -CONR"-, -NR'CO-, -NR'SO_2-, -SO_2NR"-, -CH(OH)-, -CR'(OH)-, -CR'(O-alk)-, -N-alk-, aryl, cycloalkyl, heteroaryl, heterocycloalkyl etc., and the term "alk" includes straight or branched alkyl, straight or branched alkenyl and straight or branched alkynyl; R' is H or lower alk, i.e. C_1-C_6; R" is as defined below;

-B- is absent or selected from the group consisting of:

H, alkyl, straight or branched alkyl, alkenyl (straight or branched), alkynyl (straight or branched), aryl, cycloalkyl, heteroaryl, heterocycloalkyl, alkyloxyalkyl, alkylaminoalkyl,

-C is absent or selected from the group consisting of:

-H, -OH, -NR"R"', -CONR"R"',-COO-, -COOR", -OCOR", -COR", -SO_2NR"R"', -SH, -S-S-alk, -NHCOR", -NR"COR"', $NHSO_2$R", -$NHCONH_2$, -NH-CN, -F, -Cl, -Br, -I; -SCF_3, -CF_3, -OCF_3, -SCH_3, -SR", -CN, -$N(CN)_2$,-NO_2, -OCH_3, -OR', -NH_2, -NHMe, -NHAlk, -NMe_2, -$Nalk_2$, -NMeAlk, -$N(Alk)_3^+$, heteroaryl, heterocycloalkyl, -$PO(OH)NH_2$, -SO_2OH, -COOH

and R" and/or R"' has the same meaning as given for B above optionally substituted with one or more C;

in those cases where a compound has two or more R^1 in positions adjacent to each other the –A- and/or –B- elements from the two individual R^1 may form a cyclic ring system;

in those cases where B is absent R^1 is –A-C or –A and in those cases where C is absent R^1 is –A-B or –A;

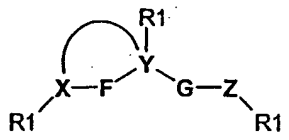
in some cases, A may be absent and then –R^1 is –B-C or –C, and B may be substituted one or more times with C, which may be the same or different;

the total number of atoms (X+F+Y+G+Z) excluding hydrogen atoms is at the most 25;

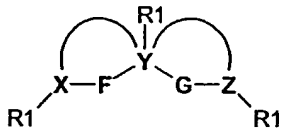
the total number of heteroatoms in (X+F+Y+G+Z) is at the most 6; and

the size of a ring is at the most 14 atoms, preferably 5 or 6 atoms.

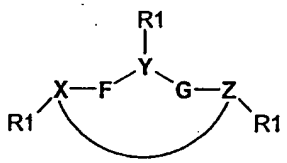
As mentioned above X, Y and/or Z may fuse to form one or more rings. Thus, X-F-Y may be part of a heterocyclyl ring system:



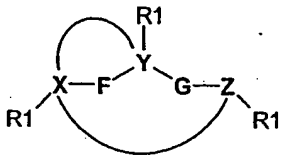
Alternatively, X-F-Y and Y-G-Z may be part of heterocyclyl ring systems:



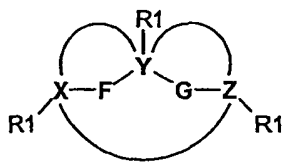
X-F-Y-G-Z may also be part of heterocyclyl ring systems:



X-F-Y and X-F-Y-G-Z may be part of heterocyclyl ring systems:



Furthermore, X-F-Y and Y-G-Z and X-F-Y-G-Z may be part of heterocyclyl ring systems:



In the present context, the term "alkyl" is intended to indicate a branched or straight-chain, saturated chemical group containing 1-15 such as, e.g. 1–12, 1-10, preferably 1-8, in particular 1-6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, sec. butyl, tert. butyl, pentyl, isopentyl, hexyl, isohexyl, heptyl etc.

The term "alkenyl" is intended to indicate an unsaturated alkyl group having one or more double bonds.

The term "alkynyl" is intended to indicate an unsaturated alkyl group having one or more triple bonds.

The term "cycloalkyl" is intended to denote a cyclic, saturated alkyl group of 3-7 carbon atoms.

The term "cycloalkenyl" is intended to denote a cyclic, unsaturated alkyl group of 5-7 carbon atoms having one or more double bonds.

The term "aryl" is intended to denote an aromatic (unsaturated), typically 6-membered, ring, which may be a single ring (e.g. phenyl) or fused with other 5- or 6-membered rings (e.g. naphthyl or anthracyl).

The term "alkoxy" is intended to indicate the group alkyl-O-.

The term "amino" is intended to indicate the group –NR''R''' where R'' and R''' which are the same or different, have the same meaning as R˜ in formula I. In a primary amine group, both R'' and R''' are hydrogen, whereas in a secondary amino group, either but not both R'' and R''' is hydrogen. In a tertiary amino group neither of R'' and R''' is hydrogen. R'' and R''' may also be fused to form a ring.

The term "ester" is intended to indicate the group COO-R'', where R'' is as indicated above except hydrogen, -OCOR˜, or a sulphonic acid ester or a phosphonic acid ester.

In the formula I above it is contemplated that if the valency of the heteroatoms F and/or G is more than 2 then further X, Y and/or Z groups may be present adjacent to the F and/or G groups.

Libraries containing specific types of test compounds

The present invention relates to libraries containing three or more test compounds. Typically, a library based on structural information from biological target molecules contains from about 3 to about 20 compounds and typically there are from about 20 to about 100 or from about 100 to about 10,000 compounds in more diverse libraries suitable for probing the vicinity of the metal-ion binding site for secondary interactions in the biological target molecule.

The aim of using a library, i.e. a collection of test compounds, is in a relatively easy and fast manner to identify the most suitable compound among a number of compounds. Normally it is a difficult task to predict which compound is the preferred one for a given biological target molecule and, therefore it is an advantage to use an approach by which a number of compounds are tested in a relatively easy and fast manner. Normally, the testing of the library is performed in suitable *in vitro* test (i.e. binding affinity test signal transduction test etc.) and then a proper selection or choice of compound(s) can be made for the *in vivo* testing. However, there may be situations where the library has a relatively small size (e.g. up to 10 compounds), which makes it possible to perform *in vivo* testing without any *in vitro* testing.

The use of a library of test compounds makes it possible to use compounds in which a systematic variation has been introduced (i.e. the position of a functional group and/or the length of a spacer arm etc.).

A library according to the invention may contain chemical compounds having a specific characteristic feature in common or it may contain chemical compounds representing a broad diversity of chemical functional groups and/or chemical structures. The chemical compounds of a library may also have a basic common structural element such as, e.g., 2.2'-bipyridine.

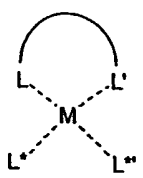
Accordingly, a library of the present invention may, e.g., contain

1. Test compounds having the same chemical functional group, C.

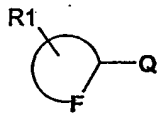
2. Test compounds having the same or almost same spacer moiety, B, in order to establish a distance from the heteroatom containing skeleton to the chemical functional group; in such a library, the chemical functional group may be the same or different.
3. Test compounds which in principle are prepared by the same method and/or which have the same kind of attachment, A, to the basic common structural element.
4. Test compounds which are capable of establishing a non-covalent interaction.
5. Test compound which are capable of establishing an irreversible or slowly reversible interaction.
6. Different test compounds which are chelated with the same metal ion.
7. The same test compound that is chelated with different metal ions.

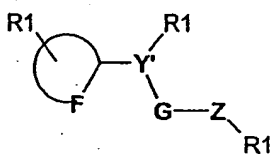
Libraries containing chemical compounds of the following general formulas are of specific interest in the present context. The individual compounds mentioned in the following as a part of a library may of course also be employed as separate compounds according to the present invention. Libraries are especially useful in step 2 and/or 7 of the target validation process (cf. page 7-8 herein).

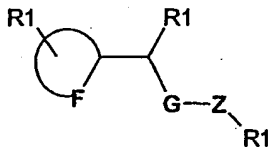
The following formulas are based on the formula I above and F and/or G have the same meaning as indicated above, i.e. F and/or G are heteroatoms. Q is a structural element containing a heteroatom, L——L' represent the heteroatoms, M represents a metal ion, L* and/or L*' are leaving groups such as, e.g. H_2O, DMSO, Cl^-, triflate etc.. A circle indicates a cyclic alkyl, alkenyl, aryl, heteroalkyl, heteroalkenyl, heteroalkynyl or heteroaryl ring having from 3-7 atoms in the ring. R^1 has the same meaning as indicated above and, when more than one R^1 is present they may be the same or different. If no specific position is given for the radical, the radical may be placed anywhere in the cyclic system and there may also be as many radicals as there is positions possible in the structure. Other symbols employed in the formulas below have the same meaning as given under formula I above. In the formulas below, the structure of the compounds are given in different structure levels. First it is given in a very general form and then in more and more specific forms. It should be noted that the invention is not limited to structures given below; these only serve to illustrate the idea of the invention and representative structures suitable for use according to the invention.

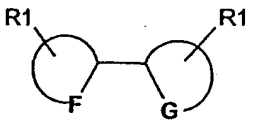


More specifically, a library according to the present invention comprises test compounds, which have one of the following structures. Y' is the remainder of the group Y which also includes Y' being absent, i.e. G being directly linked to the ring. The coordinating atom F is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom G is either included in a 5- or 6-membered aromatic, unsaturated or saturated ring or an open chain. Preferably, F is N, O or S; and G is N, O or S:

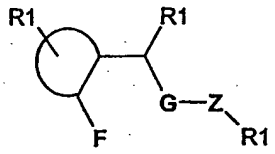


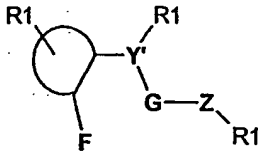


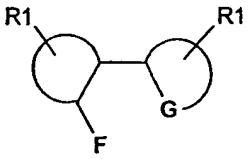




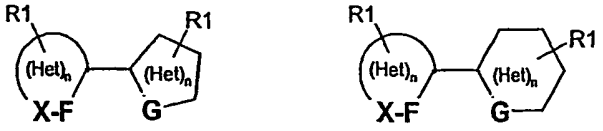
In the following subclass the coordinating atom F is appended to an aromatic, unsaturated or saturated 5- or 6-memebered ring. Preferably, F is N, O or S; and G is N, O or S.



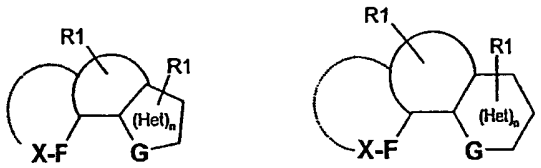




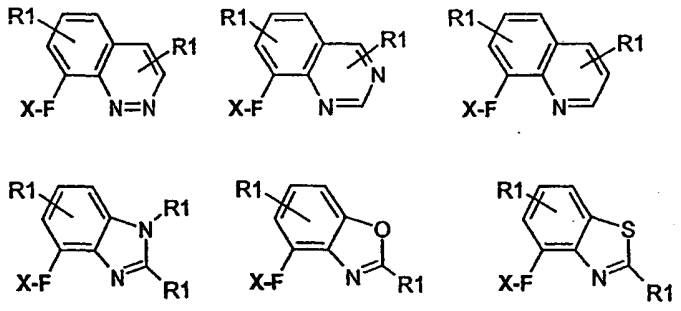
In the following biheterocyclyl subclass the coordinating atom G is included in a 5-or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and four heteroatoms and the coordinating atom F contained within an aromatic, unsaturated or saturated 5- or 6-memebered heterocycle containing between one and four heteroatoms. Preferably, F is N, O or S; and G is N, O or S.



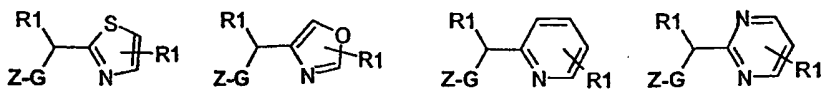
In the following subclass the coordinating atom G is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom F appended to an annelated aromatic, unsaturated or saturated 5- or 6-memebered ring. X-F can optionally be included in a fused ring as indicated by the dashed line. Preferably, F is N, O or S; and G is N, O or S.



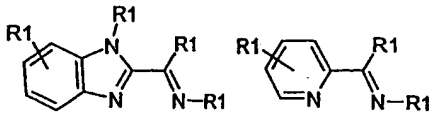
The annelated derivatives may be substituted with one or more R^1 moieties. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, pentasubstituted derivatives.



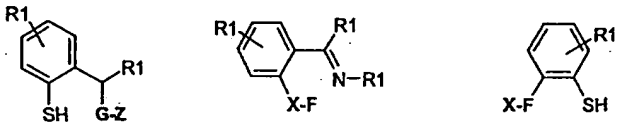
Suitable heterocyclic coordinating rings could be appended with coordinating moieties G to produce other chelating scaffolds containing one or more R1 groups.



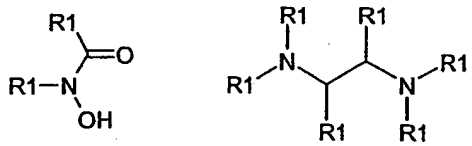
Typical coordinating scaffolds of this type are imine moieties appended to coordinating heterocycles.



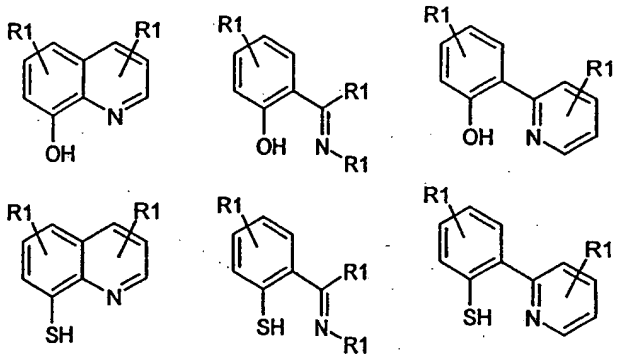
Alternatively, the coordinating groups, e.g. thiol and imine, may be attached to a ring moiety containing one or more R1 groups.



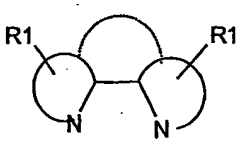
Other suitable open-chain chelating scaffolds are hydroxamic acids or 1,2-diamine coordinating moieties.



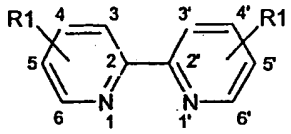
Chelator scaffolds containing one or more R1 groups of particular value are:



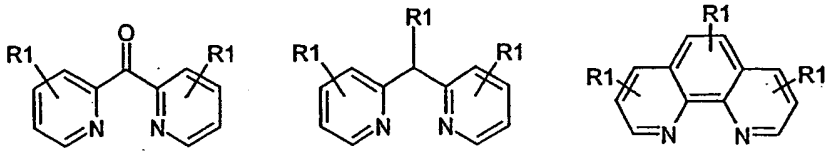
Useful nitrogen containing biheterocyclyl chelator scaffolds of particular interest are:



and, especially, pyridine containing systems of the following type



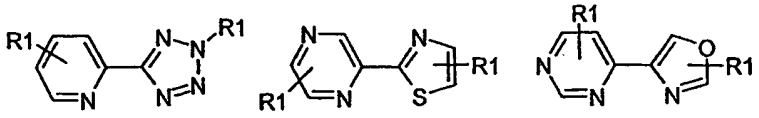
Other useful pyridyl-containg systems are systems such as

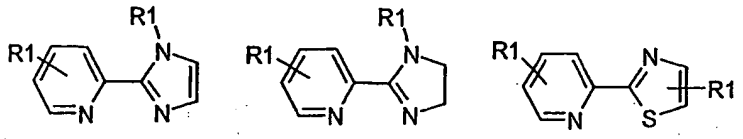


2-pyridyl systems may also be connected to other six-membered nitrogen containing rings having one nitrogen adjacent to the connecting bond, such as

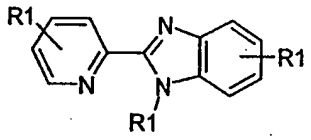
Non-pyridyl six-membered nitrogen containing aromatic rings may also be coupled to another non-pyridyl six-membered nitrogen containing ring where both ring systems having one nitrogen adjacent to the connecting bond, form useful scaffolds

The following biheterocyclyl derivatives may be substituted with one or more R^1 moieties. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, pentasubstituted biheterocyclyl derivatives. The biheterocyclyl system may be symmetric or asymmetric and they may be symmetricly or asymmetricly substituted with R1 groups.

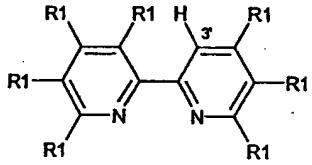




The 5-membered ring may also be annelated with e.g. a benzene ring.

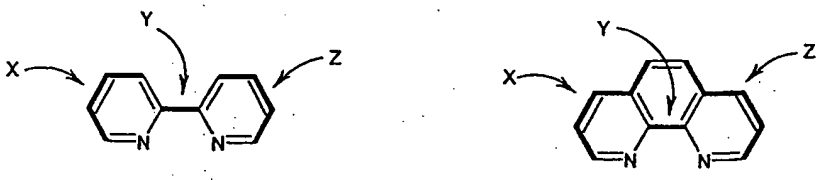


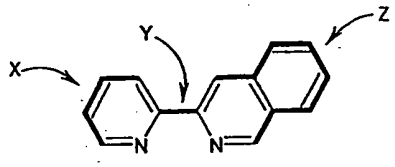
In the figure below, 2,2'-bipyridine is given as an example on a common basic structural element for chemical compounds in a library of the invention, i.e. the 2,2'-bipyridine here functions as the chelator skeleton.

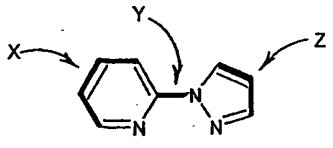


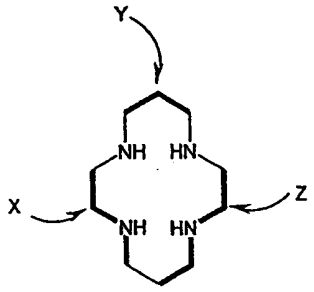
The chemical exemplifications and functionalisation principles given on this skeleton can be applied in analogous manner for other scaffolds with proper adjustments for adoption of suitable chemical routes for the different chelator systems, i.e. Che-R^1 or more specifically Che-A-B-C, wherein Che constitutes the different chelating scaffolds derived from Formula I and described above optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups.

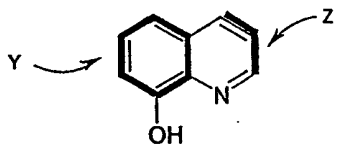
In the following are given some specific structures in which the various elements X, Y and Z are marked with bold.



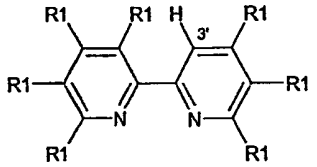








Thus, a suitable library according to the present invention is a library, that has 2,2'-bipyridine as the same basic structural element. In one embodiment this basic structural element is



Formula II

The construction of libraries according to this invention will be exemplified by the use of 2,2'-bipyridines with no intention to exclude other chelating scaffolds including the general Che-R^1 / Che-A-B-C, wherein Che constitutes the different chelating scaffolds optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups.

Accordingly, the 2,2'-bipyridines of a library according to the invention is normally substituted with one or more functional groups. Thus, a library of the present invention may contain mono-, di-, tri-, tetra-, penta-, hexa- or heptasubstituted bipyridines. The di-, tetra- and/or hexasubstituted bipyridines may be symmetric or asymmetric substituted bipyridines. Normally, up to 4 or at the most 5 substituents are present on the 2,2'-

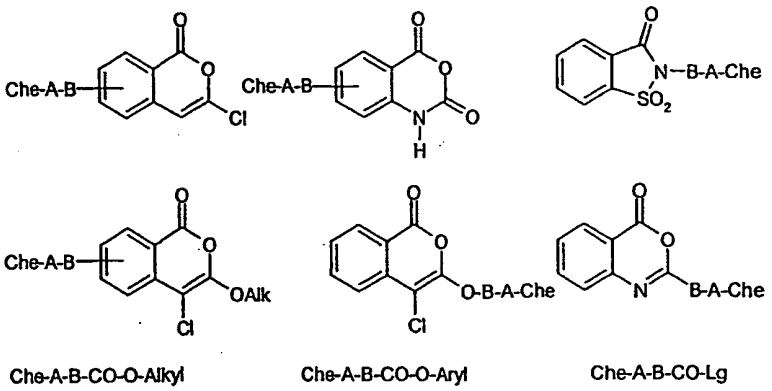
bipyridine skeleton. As seen from the formula II above, the position 3' is preferably substituted with a hydrogen atom.

Libraries of metal chelators to ascertain an irreversible or a slowly reversible binding

As mentioned above the further optimisation for the interaction between a test compound and a biological target molecule involves establishment of secondary interactions to functional groups such as, e.g. amino acid residues in the vicinity of the metal-ion binding site of the biological target molecule. In contrast to e.g. test compounds suitable for use in a drug discovery process, test compounds suitable for use in a target validation process may advantageously form a covalent bond as a secondary interaction. As way of illustration, Che-R^1 or more specifically Che-A-B-C, wherein Che constitutes the different chelating scaffolds derived from Formula I and described above is optionally substituted further with one or more, the same or different, R^1 or more specifically A-B-C groups. B is optionally substituted further with one or two, the same or different, C, the following structures for C are important in order for the (optimised) test compounds to establish a covalent (irreversible or slowly reversible) bond with e.g. an amino acid side chain in the biological target molecule. The reactivity of the C group can be modified depending on the biological system to be investigated, e.g. less reactive groups are used in *in vivo* settings whereas more reactive moieties might also be useful in for example *in vitro* cell systems.

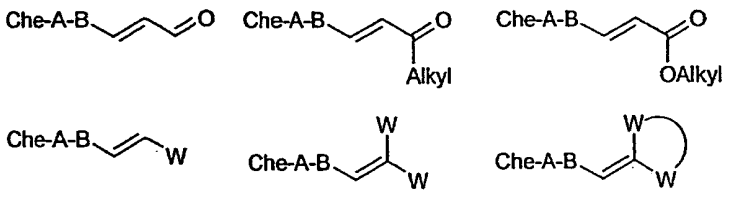
Various reactive groups can be appended as "C" to the metal chelator to ascertain an irreversible or a slowly reversible binding to an additional amino acid side chain in the biological target molecule containing a compatible reactive functional group such as, but not limited thereto, –SH, –OH, or NH_2 in the vicinity of the metal-ion binding site. Such reactive functional groups could be endogenous or mutationally introduced amino acid side-chains. Neighbouring groups on B capable of providing anchimeric assistance might modify the reactivity of these reactive groups C.

The reactive groups (selected from C) can be acylating reagents of suitable reactivity as shown below that could be appended to the metal chelator (Che as described in Formula I and the more specific examples detailed thereafter) via a spacer group (-A-B-) attached at the point indicated in the formulas with examples on –C. Lg denotes a suitable leaving group.

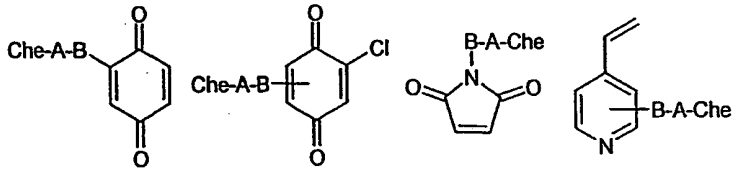


Che-A-B-CO-O-Alkyl Che-A-B-CO-O-Aryl Che-A-B-CO-Lg

Alternatively, various Michael acceptors can be appended to the metal-ion chelator. Some specific examples of a conjugated aldehyde, ketone or a conjugated carboxylic acid derivative are indicated, but the double bond could also be attached to other suitable electron withdrawing groups (W) including cyano, carboxamide, nitro, sufonyl, sulfoxide and pyridine.



The electron withdrawing groups can also be incorporated in a ring structure as specifically exemplified by the N-maleimide and quinone derivatives.



Alternatively alkylating groups with varying reactivity will be useful; e.g.

Che-A-B-NH-CO-CH_2-Lg

Che-A-B-CO-CH_2-Lg

Che-A-B-CH_2-Lg

wherein Lg being Br, Cl, F or other suitable leaving groups, for forming a covalent bond with a reactive functional group such as –SH, -OH, or NH_2 in the vicinity of the metal-ion binding site.

Additional useful reactive groups can be selected from

Che-A-B-$B(OH)_2$

Che-A-B-CO-CF_3

Che-A-B-CHO

Che-A-B-C=N-Alkyl

Reactive groups capable of reacting with –SH moieties could be selected from the following

Che-A-B-SH

Che-A-B-S-S-Alkyl Che-A-B-S-S-Aryl Che-A-B-S-S-Heteroaryl

Che-A-B-S-SO_2-Alkyl

Che-A-B-S-SO_3^-

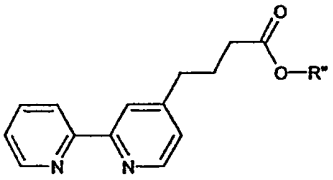
Cationic C groups such as amines, guanidines, amidines, ammonium, sulphonium or phosphonium ions might be useful to provide strong ionic interactions with negatively charged amino acids in the biological target molecule.

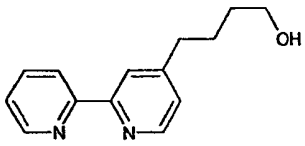
Anionic C groups such as –COOH, –SO_3H, –$PO(OH)NH_2$, tetrazoles or enoles, might be useful to provide strong ionic interactions with positively charged amino acids in the biological target molecule.

In the following are given examples of suitable test compounds as elements in a library for covalent attachment to a biological target molecule. The individual compounds mentioned in the following as a part of a library may of course also be employed as separate compounds according to the present invention. Some of the structures given below are based on 2,2'-bipyridine substituted with an alkyl or alkenyl chain. As mentioned above, the 2,2'-bipyridine structure is given as an example of a Che group and should not in any way limit the invention thereto. The alkyl or alkenyl chain has the function of separating e.g. the thiol group from the 2,2'-bipyridine structural element in such a manner that the two heteroatoms of the 2,2'-bipyridine moiety are able to interact with the metal ion in question and subsequently with the engineered metal-ion site in the biological target

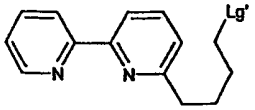
molecule and the thiol group is capable of interacting with e.g. a Cys residue present in the vicinity of the metal-ion site of the biological target molecule. It is anticipated that the length of the alkyl or alkenyl chain may be from C_1 to C_{10} such as, e.g. from C_1 to C_5; in other words, a library of the invention is not limited to the structures given below. Some of the examples are also useful as intermediates in the preparation of other reactive C groups mentioned above. The bipyridine structural element may of course have one or more further substituents such as one or more, the same or different, R^1.

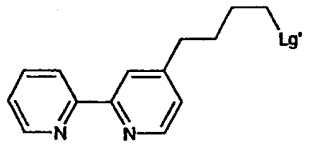
An example of carboxylic acid esters useful as test compounds and as intermediates for preparing other test compounds may be obtained by treatment of 4-methyl-2,2'-bipyridyl with LDA (lithium diisopropyl amide) followed by a reaction with ethyl 3-bromopropionate (R" being ethyl). The ester can be reduced with e.g. LAH (lithium aluminium hydride) to form the corresponding alcohol useful as intermediate in the preparation of other test compounds.

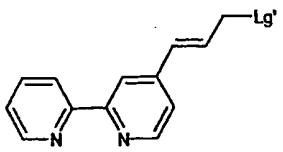




Other relevant test compounds for use in a library and for use as intermediates for preparation of other test compounds of the present invention are exemplified by the following structures, wherein Lg' is a leaving group such as – but not limited thereto – triflate, mesylate, halogen except fluorine etc.



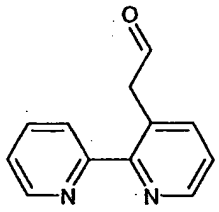


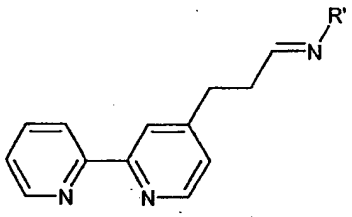


Alkylating agents, having Lg' as a leaving group, of the exemplified structures given above may be obtained from the corresponding alcohols (Lg'=OH in the above formulas) by reaction with appropriate reagents such as $SOCl_2$, $POCl_3$, PBr_3, HBr, etc to give the halogen derivatives (Lg'=Cl, Br). Alternatively, leaving groups of the type Lg' = OSO_2R' may be obtained by reaction of the formed alcohol with tosyl chloride, mesyl chloride, triflate anhydride or the like. For example, the test compounds with isocyanate (Lg'=NCO in the above formulas) as reactive group may be obtained from the corresponding bromide (Lg'=Br) by reaction with sodium cyanate.

By reacting an alkyl halide (Lg'=Br) compound with sodium maleimide the corresponding Michael acceptor, an alkyl N- maleimide, is obtained.

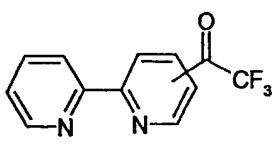
Test compounds containing an aldehyde or imine group (R' being substituted or unsubstituted alkyl or aryl groups) are also suitable for use in a library according to the present invention. The aldehydes are also useful as intermediates.



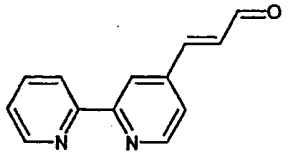


The aldehyde can be obtained by reduction of the appropriate ester, prepared from 3-methyl-2,2'-bipyridyl treated with LDA (lithium diisopropyl amide) followed by reaction with ethyl bromoacetate, with DIBAL (diisobutylaluminium hydride). Alternatively the ester may be reduced with LAH (lithium aluminiumhydride) to form the alcohol, followed by an oxidation to the aldehyde using e.g. Swern conditions with dimethyl sulfoxide and dicyclohexylcarbodiimide.

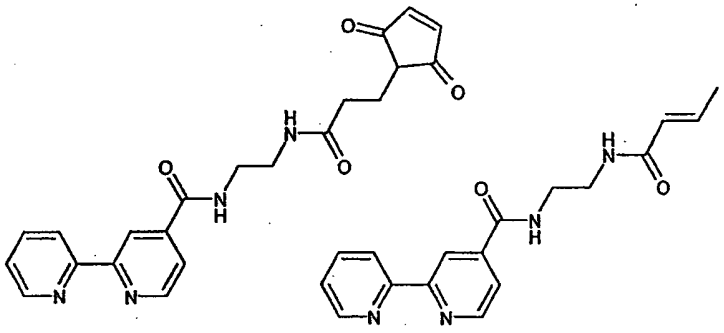
Test compounds containing a trifluoromethyl group can be synthesised from the corresponding aldehyde dy reaction with TMS-CF_3 and TBAF, followed by Swern oxidation (Sammakia *et al.*, *J. Org. Chem.*, 2000, *65*, 974-978.).



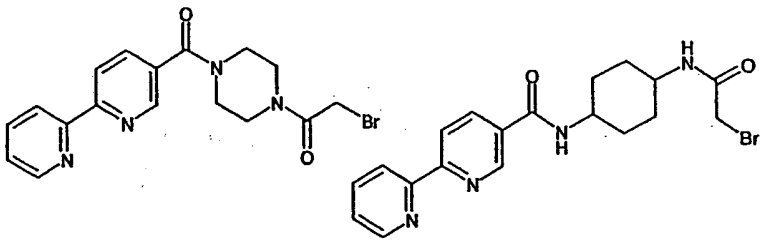
The conjugated Michael acceptor shown below may be obtained by reacting 4-formyl-2,2'-bipyridyl with vinyl magnesium bromide followed by rearrangement with acid to produce the allylic alcohol that is oxidized with manganese dioxide.



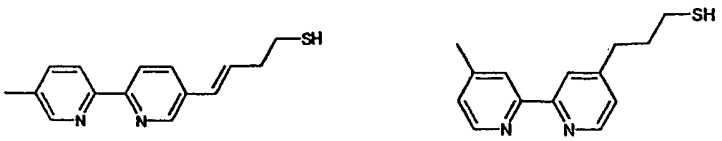
Alternatively, the B-linker can be designed via coupling of Che-COOH with diamines or amino acids as illustrated for a couple of Michael acceptors as C-moieties.



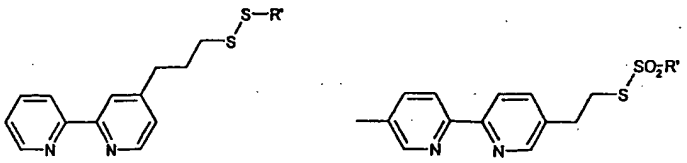
The use of the same type of peptiderigic B-linkers are illustrated with alkylating C-moieties.



The thiol compounds illustrated below may be obtained from the corresponding alkyl halide by reaction with NaSH.

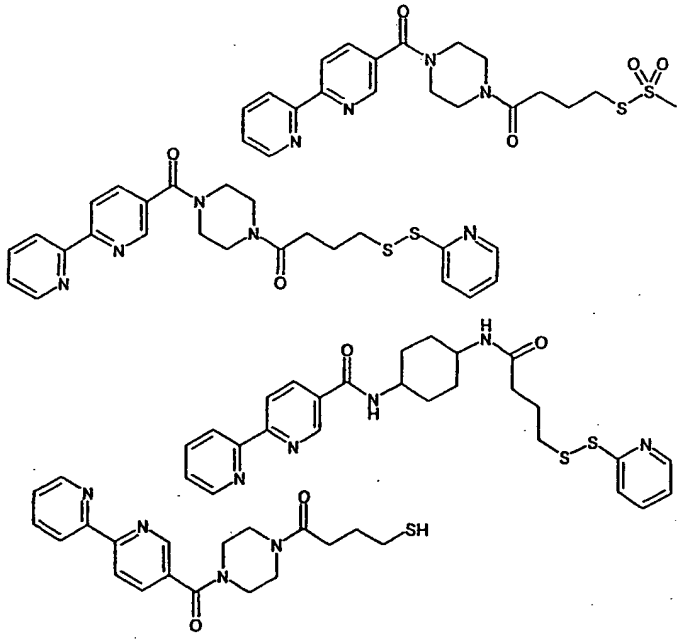


The following compounds may be obtained from the corresponding mercaptans. The unsymmetrical disulfide is made by treatment of the mercaptan with diethyl azodicarboxylate to give an adduct that is reacted with another mercaptan R'-SH.

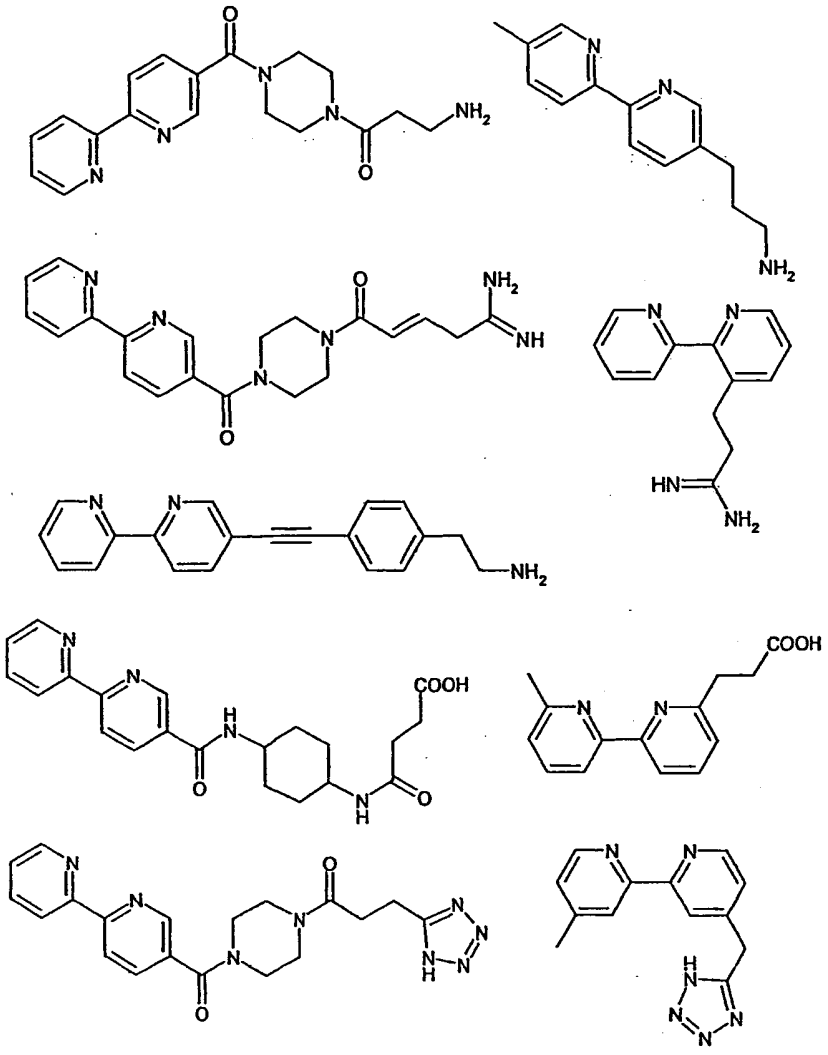


wherein R' is substituted or unsubstituted alkyl or aryl.

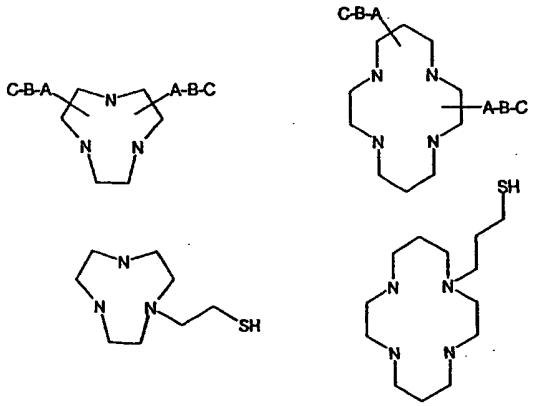
Other such useful thiol derivatives or thiol-reacting C-groups can be constructed by the peptidergic B-linkers.



Libraries containing test compounds designed for establishment of a non-covalent interaction such as, e.g., an ionic interaction may comprise test compounds of the following formulas:



Other libraries may also be designed such as, e.g., libraries of macrocyclic test compounds:



For the structures given above, the same considerations as given above under other structures apply (e.g. with respect to side chain, substituent, introduction of a catalytic function etc.).

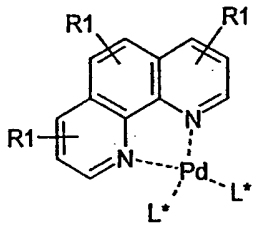
Metal atoms or ions forming the complex with the heteroalkyl or heterocyclyl moiety in the test compounds may be selected from metal atoms or ions which have been tested for or are used for pharmaceutical purposes.

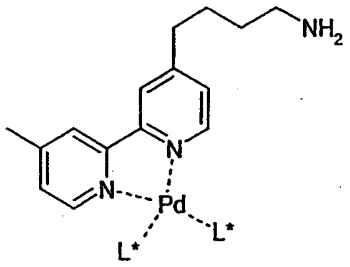
Such metal atoms or ions belong to the groups denoted light metals, transition metals, post-transition metals or semi-metals (according to the periodic system).
Thus the metal ion is selected from the group consisting of aluminium, antimony, arsenic, astatine, barium, beryllium, bismuth, boron, cadmium, calcium, cerium, caesium, chromium, cobalt, copper, dysprosium, erbium, europium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, iron, lanthanum, lead, lutetium, magnesium, manganese, mercury, molybdenum, neodymium, nickel, niobium, osmium, palladium, platinum, polonium, praseodymium, promethium, rhenium, rhodium, rubidium, ruthenium, samarium, scandium, selenium, silicon, silver, strontium, tantalum, technetium, tellurium, terbium, thallium, thorium, thulium, tin, titanium, tungsten, vanadium, ytterbium, yttrium, zinc, zirconium, and oxidation states and isotopes thereof; in particular aluminium, antimony, barium, bismuth, calcium, chromium, cobalt, copper, europium, gadolinium, gallium, germanium, gold, indium, iron, lutetium, manganese, magnesium, nickel, osmium, palladium, platinum, rhenium, rhodium, rubidium, ruthenium, samarium, silver, strontium, technetium, terbium, thallium, thorium, tin, yttrium, zinc, and oxidation states or isotopes thereof; in particular calcium, cobalt, copper, europium, iron, magnesium, manganese, nickel, palladium, platinum, ruthenium, samarium, terbium and zinc (and oxidation states or isotopes thereof, preferably cobalt (II, III), copper (I, II), nickel (II, III), zinc (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

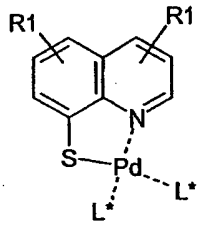
For the present purpose, a particularly favourable test compound is a chelate between any of the test compounds of the formulas mentioned above and any of the metal atoms or ions mentioned above. In particular chelates between any of the test compounds and any atom or ion of Ru, Pt, Tc, Rh, Pd, Cu, Zn, Co and/or Ni are of interest in a target validation process according to the invention. A library of the invention suitable for use in a target validation process is therefore a library comprising either the non-chelated test compounds or the chelated test compounds.

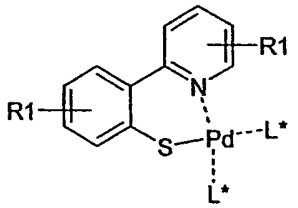
Typically, chelated test compound libraries comprise metals such as Pd and Pt forming more or less covalently bond complexes whereas non-chelated test compound libraries utilise endogenously available ions such as Zn or Cu in test preparations or animals which could have been spiked with additional non-toxic amounts of such metal ions.

Examples of chelated test compounds having palladium tightly bound to the chelating scaffold are









Normally, the chelator is complexed with a metal ion before subjecting the test compound to the metal-ion binding site in the biological target molecule. The preparation of such complexes is well-known to persons skilled in the art. However, there may be situations where the complex is formed relatively easy and then the complex may be formed *in situ* immediately before or at the same time as the testing is performed.

As appears from the examples herein, chemical diverse libraries consisting of various chelating moieties (Che) containing different spacers (A and B moieties) and functionalities (C moities) can be produced and be tested with different metal ions including covalently bound (e.g. Pt) metal ions and bind to different endogenous or engineered binding sites while exhibiting structure-related effects on receptor ligand-induced responses or receptor ligand binding.

Preparation of genetically modified test animals in which the metal ion site engineered biological target molecule is introduced into the animal

The test animals are normally animals, which have been genetically modified to express a silent metal ion site in a biological target molecule, which for example could be a metal-ion site engineered version of their own endogenous biologically target molecule or it could for example be a the human version of this molecule. The advantages by using animals, which have been genetically modified, are that the animal is otherwise normal but express a biological target molecule which can be activated or inactivated by a specific drug-like substance, i.e. a test compound which has been optimised for selective interaction with the modified biological target molecule (Jackson I. J. and Abbott C. M., (2000) "Mouse genetics and transgenics" Practical approach series.). Since this is performed in animal models, chemical modifications of the test compound is allowed which would not be suitable for a real drug. For example, test compounds which form covalent bonds to the biological target molecule can be used as test compounds e.g. as part of all kinds of covalent or non-covalent complexes formed with other compounds or artificial or biological macromolecules which infer desired characteristics to the test compound for example in respect of bioavailability and/or pharmacokinetics.

In some cases it may, however, not be necessary to genetically modify the animals. This applies when the biological target molecules within the animals *per se* have a suitable metal ion site.

Testing the test animals expressing metal-ion engineered biological target molecules with a suitable metal-ion chelate and looking for any effect

Administration routes, dosage forms and dosages

In principle all administration routes may be employed including the oral, parenteral, topical, rectal, vaginal, ocular, nasal etc. route.

A convenient administration route is the oral and the parenteral route. Dosage forms suitable for the oral route include solutions, dispersions, mixtures, emulsions, suspensions, tablets, capsules, sachets, powders, feeding powder, feeding mixture or drinking water, lotions, plasters, implants, etc. The dosage forms may be in the form of a single unit or it may presented in the form of multiple units, e.g. in capsules containing a

multiplicity of individual units. The units may be in the form of pelletizied feed for animal feeding.

The manufacture of such suitable dosage forms normally includes the use of one or more pharmaceutically acceptable excipients such as, e.g., fillers, binders, disintegrants, coating materials, solvents, emulsifiers, suspending agents, preservatives, stabilising agents, pH adjusting agents etc. all agents well-known to a person skilled in the art of pharmaceutical formulation. The dosage forms may be prepared in accordance with standard textbooks such as, e.g. Remington's Pharmaceutical Sciences.

The dose of a test compound depends on the specific test animal used. Normally, a daily dose is within the range of from about 1 μl/kg to about 1g/kg depending on the particular test compound in question. A person skilled in the art will know to find a suitable dose range. Often the dose range lies between 0.1 and 100 mg/kg. The dose may be given once daily or in separate doses during the day, e.g. two, three, four, five or six times daily or the test animal may have the dose at feeding time such as, e.g., together or via the feed. If the composition is in the form of a controlled release composition or in the form of an implant, the composition may be given less frequent such as, e.g. every day, every second day, every week, every month etc.

After administration of the test substances to the genetically modified animals (and often also to control animals, i.e. animals which have not been genetically modified) the animals are monitored with respect to any biochemical, physiological, pharmacological and/or behavioural change compared to the control animal.

Target validation process

Chemical compounds or selections of chemical compounds described herein are suitable for use in a target validation process. Details on a suitable target validation process is given in the following items:

1. A target validation process for testing or validation the physiological importance and/or the therapeutic of a biological target molecule, the process comprising

i) introduction of a silent metal ion site in the biological target molecule to obtain a silent metal ion engineered biological target molecule,

ii) *in vitro* testing of a test compound for its ability to bind to the introduced silent metal ion site in the silent metal ion engineered biological target molecule,

iii) optionally, chemically optimising the test compound and/or the biological target molecule to create secondary interaction(s) with chemical groups in the vicinity of the metal ion site in the silent metal ion engineered biological target molecule,

iv) optionally, repeating any of steps ii) and iii) to obtain a suitable binding affinity in the *in vitro* test,

v) optionally, chemically optimising the test compound to improve the pharmacokinetic and/or biopharmaceutical properties of the test compound,

vi) preparing a genetically modified test animal containing the silent metal ion site engineered biological target molecule,

vii) *in vivo* testing of the optionally optimised test compound in the genetically modified test animal, and monitoring the biochemical, physiological and/or behaviour parameters of the genetically modified test animal.

2. A target validation process according to item 1, wherein the biological target molecule is selected from the group consisting of proteins, polypeptides, oligopeptides, nucleic acids, carbohydrates, nucleoproteins, glycoproteins, glycolipids, lipoproteins and derivatives thereof.

3. A target validation process according to item 2, wherein the biological target molecule is a protein selected from the group consisting of membrane receptors, signal transduction proteins, scaffolding proteins, nuclear receptors, steroid receptors, intracellular receptors, transcription factors, enzymes, allosteric enzyme regulator proteins, growth factors, hormones, neuropeptides or immunoglobulins.

4. A target validation process according to item 3, wherein the protein is a membrane protein.

5. A target validation process according to item 4, wherein the biological target molecule is a membrane protein and the silent metal ion site in the biological target molecule is introduced in a ligand binding crevice of the membrane protein.

6. A target validation process according to item 4, wherein the membrane protein is an integral membrane protein.

7. A target validation process according to item 4, wherein the membrane protein comprises 1-14 transmembrane domains such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 domains.

8. A target validation process according to item 7, wherein the membrane protein is a receptor such as a tyrosine kinase receptor, e.g. a growth factor receptor such as the growth hormone, insulin, epidermal growth factor, transforming growth factor, erythropoietin, colony-stimulating factor, platelet-derived growth factor receptor or nerve growth factor receptor (TrkA or TrkB).

9. A target validation process according to item 7, wherein the membrane protein is a purinergic ion channel.

10. A target validation process according to item 7, wherein the membrane protein is a ligand-gated ion channel, such as a nicotinic acetylcholine receptor, GABA receptor, or glutamate receptor (NMDA or AMPA).

11. A target validation process according to item 7, wherein the membrane protein is a voltage-gated ion channel, such as a potassium, sodium, chloride or calcium channel.

12. A target validation process according to item 7, wherein the membrane protein is a 7TM receptor, a G-protein coupled receptor, such as the receptor for (– in brachet the receptor subtypes are mentioned): acetylcholine (m1-5), adenosine (A1-3) and other purines and purimidines (P2U and P2Y1-12), adrenalin and noradrenalin (α1A-D, α2A-D and β1-3), amylin, adrenomedullin, anaphylatoxin chemotactic factor, angiotensin (AT1A, -1B and -2), apelin, bombesin, bradykinin (1and 2), C3a, C5a, calcitonin, calcitonin gene related peptide, CD97, conopressin, corticotropin releasing factor (CRF1and -2), calcium, cannabinoid (CB1and -2), chemokines (CCR1-11, CXCR1-6, CX3CR and XCR), cholecystokinin (A-B), corticotropin-releasing factor (CRF1-2), dopamine (D1-5), eicosanoids, endothelin (A and B), fMLP, Frizzled (Fz1,2,4,5 and 7-9), GABA (B1 and B2), galanin, gastrin, gastric inhibitory peptide, glucagon , glucagon-like peptide I and II, glutamate (1-8), glycoprotein hormone (e.g. FSH, LSH, TSH, LH), growth hormone releasing hormone, growth hormone secretagogue /Ghrelin, histamine (H1-4), 5-

hydroxytryptamine (5HT1A-1F, -2A-C and -4-7), leukotriene, lysophospholipid (EDG1-4), melanocortins (MC1-5), melanin concentrating hormone (MCH 1 and 2), melatonin (ML1A and 1B), motilin, neuromedin U, neuropeptide FF (NFF1and 2), neuropeptide Y (NPY1,2,4,5 and 6), neurotensin (1 and 2), nociceptin, odor components , opiods (κ, δ, μ and x), orexins(OX1and -2) , oxytocin, parathyroid hormone/parathyroid hormone-related peptides, pheromones, platelet-activating factor, prostaglandin (EP1-4 and F2) prostacyclin, pituitary adenylate activating peptide, retinal, secretin, smoothemd, somatostatins (SSTR1-5), tachykinins (NK1-3), thrombin and other proteases acting through 7TM receptor, thromboxane, thyrotropin-releasing hormone, vasopressin (V1A, -1B and -2), vasoactive intestinal peptide, urotensin II, and virally encoded receptors (US27, US28, UL33, UL78, ORF74, U12, U51); and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned such as mas-proto-oncogene, EBI (I and II), lactrophilin, brain specific angiogenesis inhibitor (BAI1-3), EMR1, RDC1 receptor, GPR12 receptor or GPR3 receptor, and 7TM proteins coded for in the human genome but for which no endogenous ligand has yet been assigned.

13. A target validation process according to item 7, wherein the membrane protein is a transporter protein, such as, e.g. i) Na^+ cotransporters, including Na^+,Cl^- transporters, such as, e.g., GABA transporters, monoamine transporters, neutral amino acids transporters, kreatinin transporters and nucleoside transporters, and Na^+,K^+ coupled transporter such as, e.g., glutamate transporters, neutral amino acids transporters, and inositol transporters, and Na^+,glucose cotransporters, and Na^+,K^+,Cl^- cotransporters, ii) H+ coupled transporter including oligopeptide transporters and multi drug transporters, iii) antiporters, including Na^+/H^+- exchangers, anion exchangers such as, e.g., HCO_3^-/Cl^- exchangers and Na^+/Ca^+ exchangers, iv) ion-transporting ATPases including Na^+,K^+ ATPase, H^+,K^+ ATPase and Ca^{2+} ATPase and v) transporters from the ABC (ATP Binding Cassette) transporter family, including multidrug resistance related proteins and cystic fibrosis transmembrane regulators, and multidrug resistance proteins such as, e.g., P-glycoproteins, lung resistance related proteins and breast cancer resistance proteins.

14. A target validation process according to item 7, wherein the membrane protein is a multidrug resistance protein, e.g. a P-glycoprotein, multidrug resistance associated protein, drug resistance associated protein, lung resistance related protein, breast cancer resistance protein, adenosine triphosphate-binding cassette protein, Bmr, QacA or EmrAB/TolC pump.

15. A target validation process according to item 7, wherein the membrane protein is a cell adhesion molecule, e.g. NCAM, VCAM or ICAM.

16. A target validation process according to item 7, wherein the membrane protein is an enzyme such as adenylyl cyclase.

17. A target validation process according to item 6, wherein the membrane protein is an orphan receptor.

18. A target validation process according to item 1, wherein the silent metal ion site is constructed in a biological target molecule by mutating one or more amino acid residues into one or more amino acid residues capable of binding a metal ion.

19. A target validation process according to item 18, wherein the amino acid residues capable of binding a metal ion is selected from the group consisting of His, Cys, Asp, Trp and Glu residues.

20. A target validation process according to items 18 or 19, wherein silent metal ion site comprises at least two such as, e.g., at least three, at least four, at least five such as, e.g., two, three or four amino acid residues capable of binding a metal ion.

21. A target validation process according to item 20, wherein at least one of the at least two or more amino acid residues capable of binding a metal ion has been introduced in the metal ion site engineered biological target molecule by mutating an amino acid present in the biological target molecule.

22. A target validation process according to any of items 18-21, wherein the metal ion site comprises at least one Cys residue.

23. A target validation process according to item 1, wherein the silent metal ion site is constructed in a biological target molecule by mutating one or more non-natural amino acid residues into residues which in themselves are capable of binding a metal ion or which chemically can be modified to become a metal ion binding residue.

24. A target validation process according to any of the preceding items, wherein the silent metal ion site in the silent metal ion site engineered biological target molecule – when tested in an *in vitro* cell expression system– results in at the most a 20 fold such as, e.g.,

at the most 10, at the most 8, at the most 5, at the most 4, at the most 3 fold decrease in surface expression and/or affinity.

25. A target validation process according to any of the preceding items, wherein the silent metal ion site in the silent metal ion site engineered biological target molecule – when tested *in vivo* – does not alter or significantly alter the function of the endogenous biological target molecule.

26. A target validation process according to any of the preceding items, wherein the test compound is has a log K value in a range of from about 1 to about 50.

27. A target validation process according to any of the preceding items, wherein the test compound forms a chelate with a metal ion selected from the group consisting Ni, Pd, Pt, Ru and Zn including all possible oxidation steps such as, e.g., Pt(0), Pt (II), Pt (IV), Pd (0), Pd(II), Pd(IV), Rh , Ru(0), Ru(II), Ru(III), Ru(IV), Ru(VI) and Ru(VIII).

28. A target validation process according to any of the preceding items, wherein the test compound forms a chelate with a metal ion selected from the group consisting of Co, Cu Zn and Ni including the various oxidation steps.

29. A target validation process according to any of the preceding items, wherein the test compound has at least two heteroatoms, similar or different, selected from the group consisting of nitrogen (N), oxygen (O), sulphur (S), selenium (Se) and phosphorous (P).

30. A target validation method according to any of the preceding items, wherein step ii) or item 1 comprises

a) contacting the metal ion site engineered biological target molecule with a test compound which comprises a moiety for chelating a metal ion under conditions permitting non-covalent binding of the test compound to the metal ion site of the metal ion site engineered biological target molecule, and

b) detecting any change in the activity of the biological target molecule or determining the binding affinity of the test compound to the biological target molecule.

31. A target validation process according to any of the preceding items, wherein a residue, which can form a secondary chemical interaction, is introduced into the biological target molecule.

32. A target validation process according to any of the preceding items, wherein the secondary interaction created in step iii) is selected from the group consisting of

i) establishment of a covalent bond,
ii) establishment of a second metal ion bridge,
iii) establishment of a charge-charge interaction,
iv) establishment of an amino-aromatic interaction,
v) establishment of a hydrogen bond interaction,
vi) establishment of a hydrophobic interaction or pi-stacking, and

combinations thereof.

33. A target validation process according to any of the preceding items, wherein the secondary interaction created in step iii) is selected from the group consisting of

i) irreversible or slowly reversible covalent bonds,
ii) charge-charge interaction (introduction of charged groups such as ammonium, phosphonium or sulphonium groups, or ionisable groups such as amino or carboxy groups),
iii) hydrogen bond interactions (introduction of e.g. hydroxy, amino, keto amide, sulphonyl or other groups capable of interacting via formation of a hydrogen bond), iii) aromatic-aromatic interactions (introduction of an aromatic group in the test compound),
iv) cation-π interactions (introduction of cationic groups such as ammonium groups or introduction of an aromatic moiety)
v) hydrophobic interactions (introduction of hydrophobic groups),
vi) a second metal-ion bridge or anchor established through a metal-ion binding moiety on the variable part of the compound and a metal-ion binding residue on the biological target molecule, and

combinations thereof.

34. A target validation process according to item 32 or 33, wherein the secondary interaction is created through establishment of a covalent bond.

35. A target validation process according to item 34, wherein the covalent bond is established with a thiol-containing amino acid residue (e.g. Cys) or an amine-containing amino acid residue (e.g. Lys).

36. A target validation process according to any of the preceding items, wherein a residue, which can form a secondary chemical interaction with the test compound, is introduced into the biological target molecule.

37. A target validation process according to any of the preceding items comprising step v) of item 1.

38. A target validation process according to item 37, wherein the test compound is optimised for appropriate properties with respect to *in* vivo absorption, distribution metabolism and excretion, or with respect to biopharmaceutical properties such as, e.g., water solubility, lipid solubility or particle size.

39. A target validation process according to item 37, wherein the test compound is linked to a carrier.

40. A target validation process according to item 39, wherein the carrier is a macromolecular carrier of natural, synthetic or semisynthetic origin.

41. A target validation process according to item 39, wherein the carrier is a protein, an oligopeptide, a peptide, a polysaccharide, an oligosaccharide, a polyethylene glycol, a poly lactic acid, a poly glycolic acid, a poly(lactic-glycolic) acid, an acrylic acid polymer, an ethyl-vinyl acetate polymer, hyaluronic acid, gelatine, an antibody, a fragment of an antibody or the like.

42. A target validation process according to any of items 37-41, wherein the link between the test compound and the carrier is established through formation of a complex or through a covalent binding.

43. A target validation process according to any of items 37-41, wherein the link between the test compound and the carrier is established through a spacer.

44. A target validation process, wherein the genetically modified animal is an animal, which has a metal-ion site biological target molecule.

45. A target validation process, wherein the genetically modified animal is an animal into which a metal ion site engineered biological molecule has been introduced.

46. A target validation process, wherein the genetically modified animal is an animal, which expresses a metal ion site engineered biological molecule.

47. A target validation process according to any of the preceding items, wherein the genetically modified animal containing a metal ion site engineered biological target molecule is obtained by employment of a double replacement method.

48. A target validation process according to any of the preceding items, wherein the test compound, optionally chemically optimised, is administered to the genetically modified animal and biochemical, physiological and/or behaviour parameters compared are monitored.

49. A target validation process according to any of the preceding items, wherein the metal ion is one that binds to an amino acid residue containing a S, O, N, Se and/or P atom or with an aromatic amino acid residue.

50. A target validation process according to item 49, wherein the amino acid residue is selected from the group consisting of Ser, Lys, Arg, Tyr, Thr, Trp, Phe, Asp, Glu, Asn, Gln, Cys and His, in particular Asp, Glu, Cys, Trp and His.

The invention is further illustrated in the following non-limiting examples described below

Legends to figures

Figure 1 shows the generic nomenclature of 7TM receptors exemplified by family A 7TM receptors. One or two conserved key residues are highlighted in each TM: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17.

Figure 2 shows the syn, anti and direct coordination of the metal-ion in carboxylate groups. The percentage of each is shown in brackets, based on the analyses of 67 compounds from Cambridge Structural Database (Carrell et al, 1988).

Figure 3 shows the two tautomeric forms of the neutral imidazole side chain of histidine. Without metal, the NH-epsilon-2 form is predominant (80%), whereas the NH-delta-1 form predominates upon metal-binding (75%).

Figure 4 shows the geometric shapes and coordination numbers of metal complexes. The most common motifs are italicised.

Figure 5 relates to the Identification of naturally occurring metal-ion binding site in the 7TM leukotriene LTB4 receptor in Example I.1. The figure shows a whole cell competition binding experiment with COS-7 cells expressing the wild type and mutant variants of the leukotriene LTB4 receptor using [^{3}H]-LTB4 as the radioligand.
Panel A. Affinity of Cu(II), 2,2'-bipyridine and the complex therof in the wild type LTB4 receptor.
Panel B. Affinity of Cu(biprydine) in mutant forms of the LTB4 receptor in which the metal-ion binding is severely imparired.
Panel C.Helical wheel diagram illustrating the transmembrane segments of the LTB4 receptor. The two cysteine residues within the transmembrane segment III which have been identified as critical for metal-ion chelator complex binding, Cys93 and Cys97 are indicated in dark gray.

Figure 6 relates to identification of a naturally occurring metal-ion binding site in the 7TM Galanin receptor-1 in Example I.2.
Panels A-F. Mutational analysis and identification of putative Zn(II) chelating residues in the Gal-R1.
Panels G. Model of the Gal-R1 receptor with putative Zn(II) chelating residues shown in the transmembrane segments.
Panel H and I. Model for chelation of Zn(II) by GalR1 from the mutational analysis.

Figure 7 relates to identification of naturally occurring metal-ion chelator binding site in the 12TM dopamine transporter in Example I.3. The figure shows a competition analysis of uptake of [^{3}H]-dopamine in whole COS-7 cells expressing the dopamine transporter.
Panel A. Uptake of [^{3}H]-dopamine by the wild-type dopamine transporter in the presence of free metal zinc-ion and zinc in complex with the chelator 2,2'-bipyridine.
Panel B. Dopamine uptake analysis in a mutant form of the dopamine transporter, [H193K], in which binding of the metal-ion complex has been eliminated (Noregaard et al. EMBO J. (1998) 17: 4266-4273).

Panel C. Effect of metal-ion complex formation on the ability to inhibit [^{3}H]-dopamine uptake in the wild-type and [H193K] mutant dopamine transporter.

Figure 8 shows the binding of various metal-ion complexes to a library of inter-helical metal-ion sites engineered into the tachykinin NK1 receptor as described in Example II.1. COS-7 cells expressing various engineered forms of the NK1 receptor were analyzed by competition binding using [^{125}I]-Substance P as radioligand.
Panel A. IC_{50} values for the zinc and copper metal-ions and complexes thereof with the chelators, 2,2'-bipyridine and phenanthroline are presented in the table. N indicated the number of experiments performed.
Panel B. Data obtained using the chelator cyclam are presented for the NK1 mutant in which an inter-helical metal-ion site has been generated through the introduction of the HisV:05;HisVI:24 exchanges.
Panel C. A helical diagram representing the four sets of inter-helical metal-ion sites which appear in Panel A are indicated.

Figure 9 relates to re-engineering of a metal-ion chelator binding site in the 12TM dopamine transporter as described in Example II.2. Dopamine uptake was analysed in COS-7 cells expressing the wild type and mutant forms of the dopamine transporter in competition with the metal-ion chelator complex, zinc(II)-2,2'-bipyridine. The two panels show two forms of re-engineered dopamine transporters in which the ability to bind the metal-ion chelator complexes have been reconstituted following the elimination of the His193 interaction point.

Figure 10 relates to fluorescence measurement of the relative strength of a selection of chelators to chelate Zn(II) in competition with FluoZin-3 as described in Example II.3.

Figure 11 shows the structure-activity relationship of metal-ion complexes in the leukotriene LTB4 receptor as described in Example III.1.Competition binding analysis in COS-7 cells expressing the LTB4 receptor. Binding of [^{3}H]-LTB4 was analysed in the presence of various copper-ion chelator complexes.

Figure 12 shows the structure-activity relationship of antagonist metal-ion complexes in the metal-ion site engineered tachykinin NK1 receptor as described in Example III.2 Binding of [^{125}I]-Substance P was analysed in COS-7 cells expressing NK1 receptor which have been engineered to bind the zinc metal-ion. Ligand binding is presented in competiton with the zinc metal-ion, the zinc-1,10-phenanthroline complex and with other

zinc-chelator xomplexes as indicated.

Figure 13 relates to engineering of *agonistic* metal-ion bindng sites in the Beta-2 adrenergic receptor, demonstrating the importance of the specific amino acids defining the site as described in Example III.3.1.
Panel A. Agonistic metal-ion binding sites probed with either Cu(II)-(2,2'-bipyridine)3 or Cu(II)-(1,10-phenanthroline)3 demonstrating the importance of the specific amino acids composing the site in defining the potency of the sites.
Panel B. Histogram showing the observed efficacy using copper-complexes of 2,2'-bipyridine or 1,10-phenanthroline on selected engineered agonistic metal-ion sites in the Beta-2 adrenergic receptor demonstrating the importance of the observed efficacy on the specific amino acids composing the site. See also figure 20.

Figure 14 shows the structure-activity relation ship of agonistic metal-ion complexes in the metal-ion site engineered beta-2-adrenergic receptor as described in Example III.3.2
The effect of Cu(II) and copper-chelator complexes on stimulation of accumulation of intracellular cAMP was analyzed in COS-7 cells expressing the beta2-adrenoceptor.
Panel A. Washing experiment demonstrating the reversibility of the stimulatory action of the metal-ion complexes.
Panel B. Dose-response analysis of selected copper-chelator complexes on the [F289C;N312C] beta2-AR.

Figure 15 (cont. of figure 14) shows the structure-activity relation ship of agonistic metal-ion complexes in the metal-ion site engineered Beta-2-adrenergic receptor.
The effect of Cu(II)-chelator complexes on stimulation of accumulation of intracellular cAMP was analyzed in COS-7 cells expressing the beta2-adrenoceptor.
Panel C. Testing a library of Cu(II)-2,2'-Bipyridine complexes at 10 micromolar on the [[F289C]-Beta-2 Adrenergic receptor for their efficacy in stimulating cAMP. For a list of compounds see the list below.

Figure 16 shows the structure-activity relationship of antagonistic metal-ion complexes in a soluble protein, the enzyme factor VIIa as described in Example III.4. The figure shows a comparison of selected metal-ion complexes on the binding of [3H]-LTB4 and the inhibition of the enzymatic activity of the active form of Factor VII (FVIIa) in COS-7 cells expressing respectively the LTB4 receptor (Panel B) and the FVIIa (Panles A and C). For stucture of the chelators see the list below.

Figure 17 shows a structure-based optimization of metal-ion chelators for secondary interactions in the CXCR4 receptor and other biological targets as described in Example III.5 Helical wheel diagram for the CXCR4 receptor. The Asp171 residue present in the transmembrane segment IV, and which is considered a major attachment site for the binding of the cyclam chelator is shown in white on black. Positions, which in combination are proposed to constitute putative metal-ion binding sites, are high-lighted in pairs and in black on dark gray.

Figure 18 relates to affinity optimization of metal-ion chelators in the LTB-4 leukotriene receptor as described in Example III.6.
A library of approximately 500 substituted 2,2'-bipyridines were tested in competition binding with [^{3}H]-LTB4 in COS-7 cells expressing the LTB4 receptor. From this test, TM-369 was identified as having a 50-fold increased affinity compared to the unsubstituted 2,2'-bipyridine scaffold.

Figure 19 relates to probing different metal-ions in an engineered Bis-His TM-V Kappa opioids.

Figure 20 shows the dependency of amino acids defining the metal-ion site, metal-ion or metal-ion chelator on the observed efficacy in agonist metal-ion binding sites in the beta-2 adrenergic receptor.
Shown is the cAMP production in response to 100 micromolar complex, compound, free ion (10 micromolar Cu(II)) or 1 micromolar pindolol.

Figur 21 is related to Exampel III.7. The figure illustrates the establishment of increased affinity in a silent metal-ion site engineered receptor through second-site interaction obtained by side-chain modification of a stable metal-ion chelator complex to be used in a genetically modified animal. Signal transduction is determined as accumulation of [3H] Inositol triphosphat in COS-7 cells expressing either the wild-type RASSL receptor (called RO2 in the figure) or the metal-site engineered RASSL receptor (called CysVII:06 in the figure). The metal ion site is located between position TM III:08 (a natural Asp residue) and VII:06 (an engineered Cys residue). The receptors are stimulated with a constant dose of the non-peptide agonist ICI 199,441 to a sub-maximal level and inhibitory dose-response experiments are performed with preformed stable Pd(II) complex with either 4,4-dimethyl-bipyridine (chemical structure shown to the upper right and dose-response experiments in wild-type receptor and metal-ion site engineered receptor are shown in the panel to the upper left) or in complex with compound 433 (chemical structure shown to the

lower right and dose-response experiments in wild-type receptor and metal-ion site engineered receptor are shown in the panel to the lower left) – in both cases acetate (AcO) was used as leaving group.

Figures 22 and 23 show the structure-activity relationship of antagonist metal-ion complexes in the metal-ion site engineered RASSL receptor. Accumulation of [3H] Inositol triphosphat was measured in COS-7 cells expressing RASSL receptor, which have been engineered to bind the zinc metal-ion. The metal ion site is located between TM III:08 and VII:06. The receptor is stimulated with the non-peptide agonist ICI 199,441 to a sub-maximal level and inhibited with the zinc-1,10-phenanthroline complex and with the 5'-chloro substituted phenenthroline analog.

Figure 24 shows an antagonist of different metal-ions in complex with 5-chloro1,10 phenanthroline in the metal-ion site engineered RASSL receptor. Accumulation of [3H] Inositol triphosphat was measured in COS-7 cells expressing RASSL receptor, which have been engineered to bind the zinc metal-ion. The metal ion site is located between TM III:08 and VII:06. The receptor is stimulated with the non-peptide agonist ICI 199,441 to a sub-maximal level and inhibited with the 5-chloro-1,10-phenanthroline in complex with Zn(II) and in complex with Pd(II).

Figure 25 shows that Pd(II)-5-chloro1,10phenanthroline act as an antagonist in the metal-ion site engineered RASSL receptor but not in the wild type RASSL receptor. Accumulation of [3H] Inositol triphosphat was measured in COS-7 cells expressing wild type RASSL receptor and the RASSL receptor, which have been engineered to bind the metal-ion. The metal ion site is located between TM III:08 and VII:06. The receptors are stimulated with the non-peptide agonist ICI 199,441 to a sub-maximal level and inhibited with the Pd(II)-5-chloro-1,10-phenanthroline.

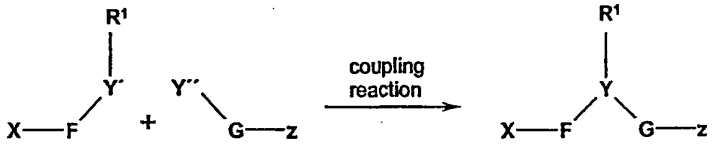
Figure 26 shows pre-incubation with Pd(II)-5-chloro1,10phenanthroline before stimulation with the non-peptide agonist ICI199.441 in the metal-ion site engineered RASSL receptor. Accumulation of [3H] Inositol triphosphat was measured in COS-7 cells expressing the RASSL receptor, which have been engineered to bind the metal-ions. The metal ion site is located between TM III:08 and VII:06. The receptor is pre-incubated with the Pd(II)-5-chlorophenanthrolin for 30 min and after repeated washing it is stimulated with the non-peptide agonist ICI 199,441.

In the following is given methods for the preparation of suitable chemical compounds for use in the present invention. The invention is further illustrated in the following non-limiting examples.

Formula I may be constructed by well-known synthetic steps involving coupling reactions, including Stille-, Suzuki-, Negishi-, Ullmann-couplings (C-C bond formations), condensation reactions, including heterocyclic ring-forming reactions, elimination reactions, cycloaddition reactions, and/or substitution reactions known from the common literature, as illustrated with some typical but non-limiting reaction schemes.

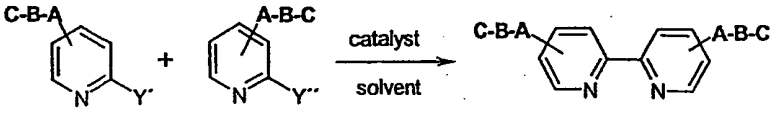
The usual considerations regarding which functional groups that are compatible with the different types of chemistries should always be taken into account when selecting synthetic routes, order of introduction of functional groups and their interconversions, etc, which accordingly will differ on a case by case basis but are evident for the skilled person.

One typical connection of coordinating moieties is depicted in Scheme I, where Y′ and Y′′ are defined such that they represent functional groups enabling coupling reactions.



Scheme I

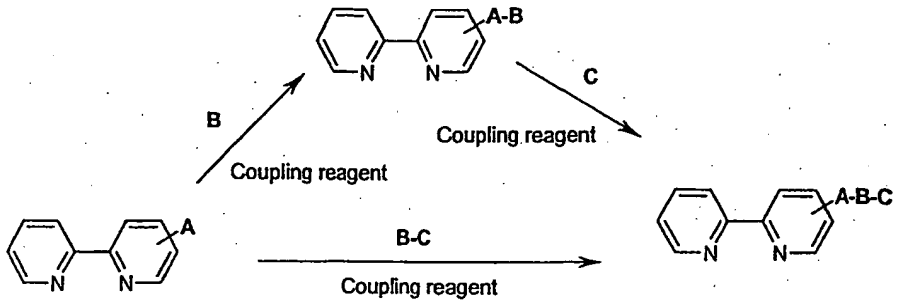
More specific descriptions of the reaction types are exemplified in Schemes II, III and IV respectively. Scheme II illustrates the C-C-bond forming reaction in the 2,2'-bipyridine series.



Reaction type I

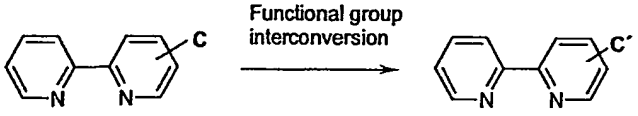
Scheme II

Modification of the chelating scaffolds exemplified by bipyridins can be made in essentially two ways, depicted in Schemes III and IV, either by coupling of an **A**-moiety with a **B**-moiety followed by **C**-moiety, or a **B-C**-moiety, or as illustrated in Scheme IV by a functional group interconversion.



Reaction type II

Scheme III

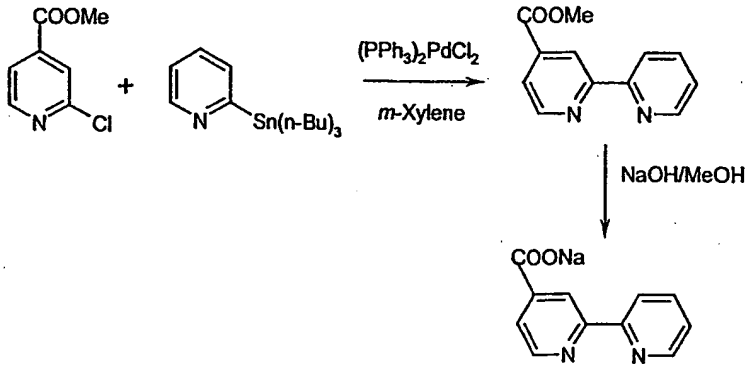


Reaction type III

Where **C** and **C'** represent a change in functionality.

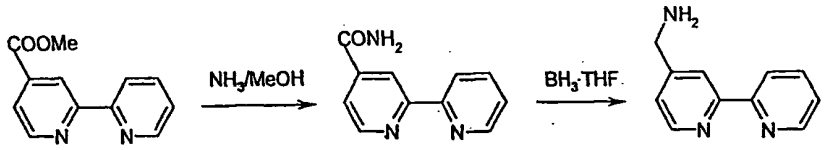
Scheme IV

Coupling of functionalised heterocyclic ring systems such as chloropyridines with trialkyl tin pyridines can be performed by the Stille coupling method, and exemplified in Scheme V.



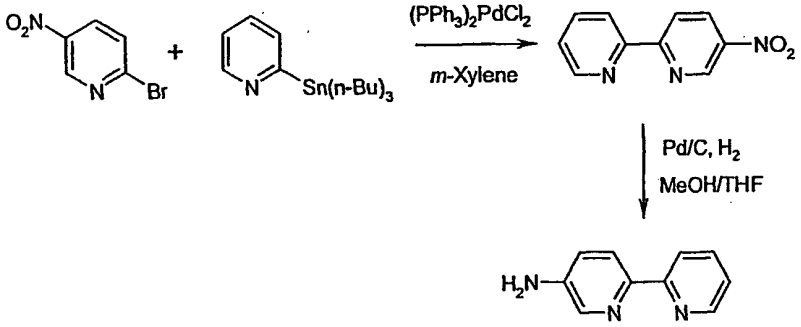
Scheme V

Typical functional group interconversions are exemplified by transforming -$COOCH_3$ into a -CH_2-NH_2 moiety as exemplified with the 2,2'.bipyridine system.



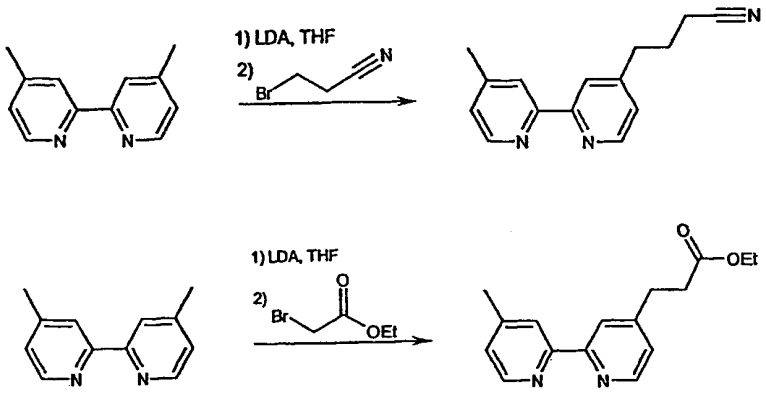
Scheme VI

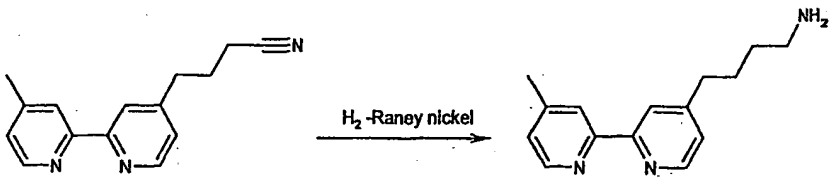
Certain other types of functionalities on the pyridine ring can accepted in the coupling reaction step as illustrated in Scheme VII.



Scheme VII

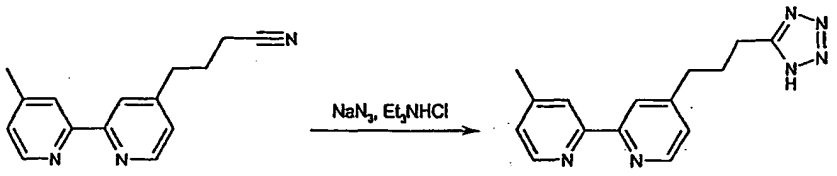
Other types of functionalisations are illustrated by the synthesis of longer chain 2,2'-bipyridyl amines from the symmetric dimethyl-2,2'- bipyridines, by generation of dimethyl-2,2 bipyridine anion with LDA followed by addition of the appropriate electrophile. Standard reduction of the nitrile yielded the desired product as outlined in Scheme VIII.





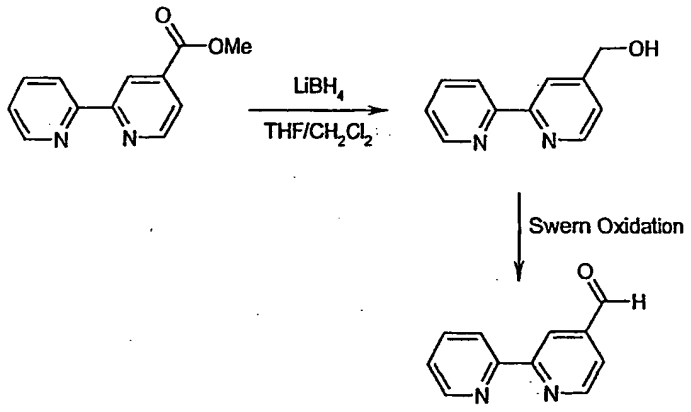
Scheme VIII

Functional group interconversions could utilise common intermediates (cf. Schemes VIII and IX) as illustrated by the bipyridine functionalised chelating scaffold



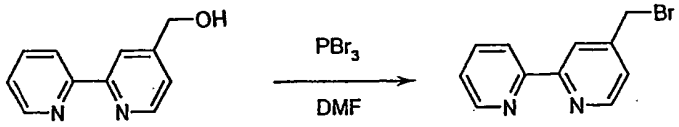
Scheme IX

Reduction of the bipyridine esters were performed by using $LiBH_4$, in DCM/THF as solvent, whereupon the corresponding alcohols were oxidised under Swern conditions to the corresponding aldehydes, as exemplified in Scheme X.



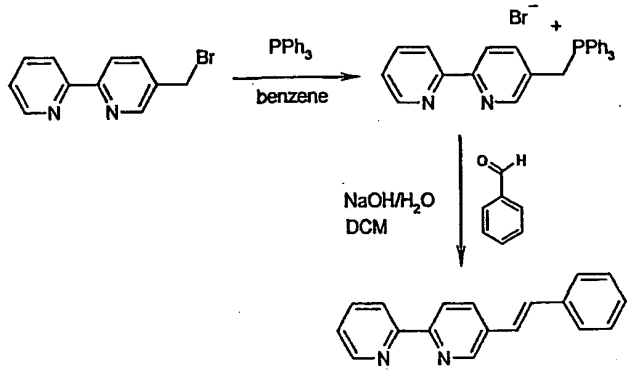
Scheme X

Functional group interconversion of the methylhydroxy functionality to the corresponding bromide can be performed by standard literature procedure as seen in Scheme XI.



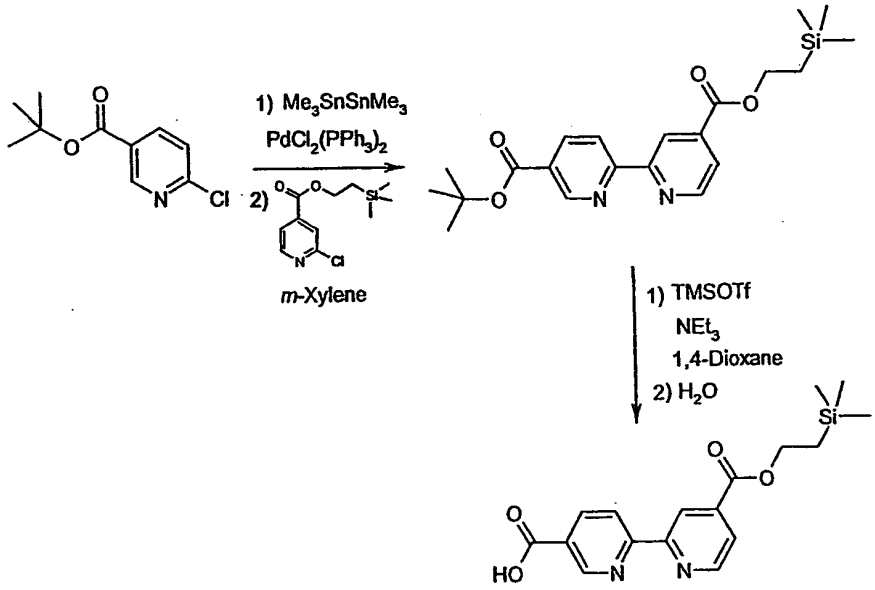
Scheme XI

The synthesis of alkenes the Wittig reaction protocol was utilised as outlined in Scheme XII.



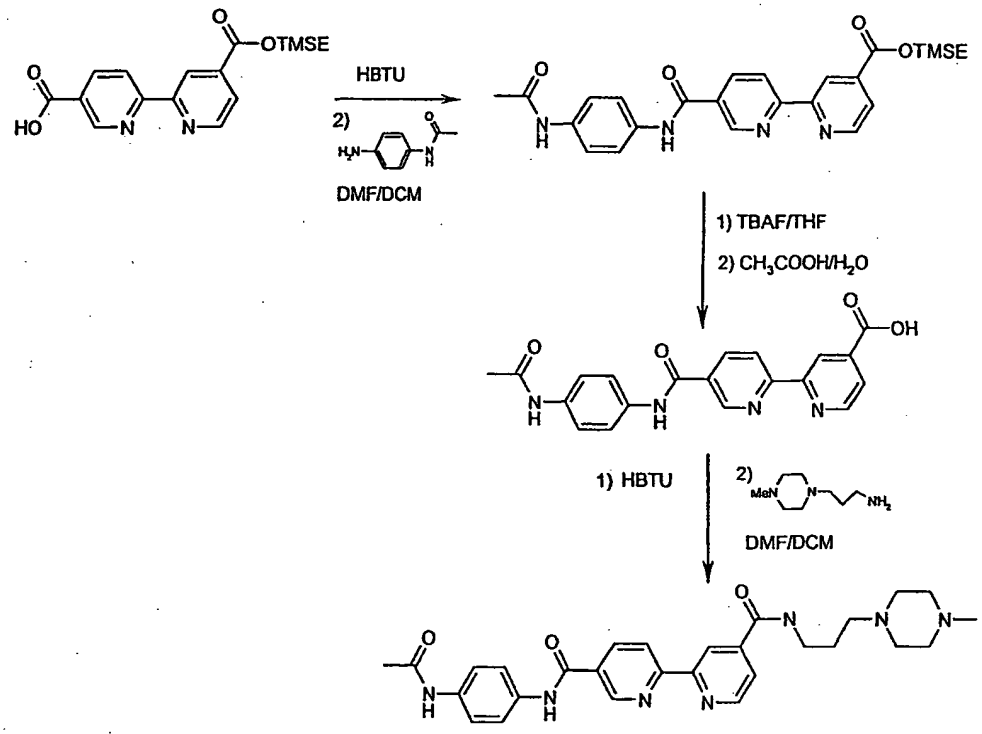
Scheme XII

Coupling of functionalised chloropyridines were performed by using $Me_3SnSnMe_3$, and thereby in situ forming the corresponding trimethyltin pyridine, which was subsequently coupled to the differently substituted chloropyridine as shown in Scheme XIII.



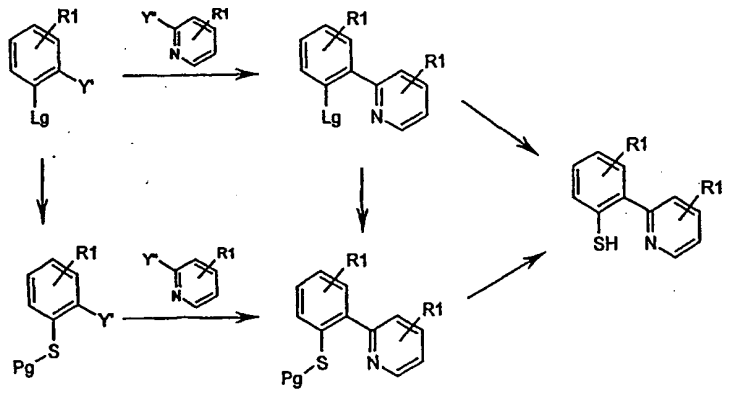
Scheme XIII

Further functionalisations of the unsymmetrically substituted bipyridines were performed by a orthogonal deprotection procedure as in Scheme XIV using standard literature procedure. Amine coupling of the free carboxyl acids can be performed by using a suitable coupling reagent.



Scheme XIV

Similarly, other chelator systems may be formed and manipulated. As an example on a chelator which have one of the coordinating atom(s) outside the ring system is 2-(2-pyridyl)thiophenol (See Scheme XV). In this case, the construction may follow different routes, i.e. the coordinating atoms may be introduced at various stages, protected or unprotected, schematically illustrated in Scheme XV.



Scheme XV

Further functionalisation of the R1-group can be made analogous to the above described procedures.

Abbreviations.

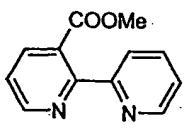
DCM	Dichloromethane
DIBAL	Diisobutylaluminum hydride
DMF	*N,N'*-Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
HBTU	*O*-Benzotriazole-1-yl-*N,N,N', N'*-tetramethyluronium hexafluorophosphate
HOBT	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
LDA	Lithium diisopropylamide
Lg	Leaving group
MS	Mass Spectrometry
NMR	Nuclear magnetic resonance
Pg	Protecting group
R.T.	Room temperature
TBAF	Tetrabutylammonium fluoride
Tf	Triflate

TFFH	Fluoro-*N,N,N'*, -tetramethylformamidinium hexafluorophosphate
THF	Tetrahydrofurane
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
TMSE	2-(Trimethylsilyl)-ethyl

General chemical procedures

All reagents/chemicals were used as received unless otherwise noted. Methyl esters of 2-chlorocarboxy pyridines were synthesised using carbonyldiimidazole. Coupling of the 2-chloromethyl carboxylates with 2-(tributyl)tin pyridine and hydrolysis of the resulting 2,2'-bipyridinemethyl esters were performed by Stille coupling according to the method described by Panetta *et al.* (*J. Org. Chem*, **1999**, *64*, 1015-1021). Coupling between the 2,2'-bipyridinesodium carboxylate and selected primary amines (example 3) were perfomed according to standard procedure. Reduction of 4-nitro-2,2'-bipyridine to the corresponding 4-amino-2,2'-bipyridine was accomplished by hydrogenation according to the method by Imperali *et al.* (*J. Org. Chem.*, **1996**, *61*, 8940-8948). All terazoles of 2,2'-bipyridnes were synthesised according to the method of Koguru *et al.* (SYNTHESIS, **1998**, 910-914). Guanidines of amino or alkylamino 2,2'-bipyridine were synthesised according to the method of Patek *et al.* (SYNTHESIS, **1994**, 579-582). Aldehydes of 2,2'-bipyridine were synthesised by reduction of 2,2'-bipyridinemethyl esters using lithiumborohydride according to the method of Uenishi *et al.* (*J. Org. Chem.*, **1993**, *58*, 4382-4388). Oxidation of the resulting methylhydroxy 2,2'-bipyridine to the corresponding aldehyde was performed according to the method of Swern *et al.* (*Tetrahedron*, **1978**, *34*, 1651-1660). All other reactions were carried out according to reported procedures.

EXAMPLE 1

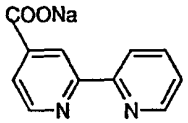


2,2'-Methyl-2,2'-bipyridine-3-carboxylate. 2-Chloronicotinic acid methylester (154.9 mmol, 26.7g) was suspended in 500ml dry *m*-xylene, in an oven dried 1000 ml two-necked round bottomed flask equipped with stirrer magnet. 2-Tributyltin pyridine (176.2 mmol, 80g) was

added and thereupon *bis*-triphenylphosphinepalladium chloride (9.6 mmol, 6.4g). The resulting mixture was heated to 130 C for 6h under N_2-atmosphere. The dark-brown mixture was then allowed to cool to ambient temperature, and the solvent was removed by evaporation *in vacuo*. The residue was mixed with dichloromethane (50 ml), and purified by column chromatography (CH_2Cl_2:EtOH, 95:5). The pure compound was retrieved as white crystals. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.74 (dd, J = 1.68, 5.1 Hz), 8.61-8.59 (m, 1H), 8.15-8.12 (m, 1H), 7.96 (dd, J = 1.71, 7.8 Hz, 1H), 7.87-7.77 (m, 1H), 7.36 (dd, J = 4.71, 9.0 Hz, 1H), 7.32-7.28 (m, 1H), 3.78 (s, 3H).

In the same manner the corresponding ethyl, propyl, isopropyl, isobutyl, *tert*-butyl, phenyl, pentafluormethyl, 9-fluorenylmethyl, 2-trimethylsilylethyl bipyridine esters (in 3, 4 or 5-position), (alkyl)aldehydes or (alkyl)nitriles are synthesised.

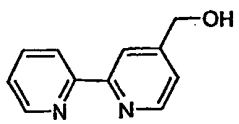
EXAMPLE 2



Sodium-2,2´-Bipyridine-4-carboxylate. Sodiumhydroxide (131.0 mmol, 5.2g) was dissolved in absolute methanol (300 ml). Bipyridine-4-carboxymethyl ester (130.7 mmol, 28g) was added and the resulting mixture was refluxed for 3h. A white precipitate formed. The mixture was allowed to cool to ambient temperature. The white precipitate was collected by filtration, and washed with ether. The mother liquor diluted with ether (150 ml), and the resulting precipitated was collected by filtration and washed with ether. The remaining solid was allowed to dry at room temperature.

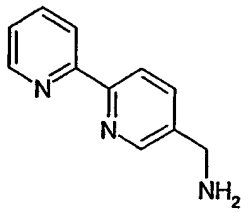
Sodium-2,2´-bipyridine-3-carboxylate and sodium-2,2´-bipyridine-5-carboxylate were prepared according to identical procedure.

EXAMPLE 3



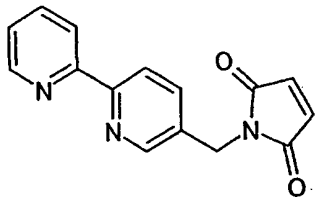
4-(Hydroxymethyl)-2,2´-bipyridine. Methyl 2,2´-bipyridine-4-carboxylate (9.34 mmol, 2.0 g) was dissolved in MeOH/DCM (5 ml/50 ml), whereupon $LiBH_4$ (18.67 mmol, 0.4 g, 2 equiv.) was added and the reaction mixture was stirred at room temperature for 3h. Another portion of $LiBH_4$ (9.33 mmol, 0.2 g, 1 equiv.) was added and the reaction mixture was stirred at room temperature for 16 h. The reaction was quenched with acetone. The solvent was removed *in vacuo* after which the solid residue was dissolved in DCM, and chromatographed on a silica column (DCM/MeOH/NH_3, 100/10/1).Yield: 92 %. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.59 (ddd, J = 0.93, 1.5, 3.96 Hz, 1H), 8.52 (dd, J = 0.57, 2.25 Hz, 1H), 8.29 (dt, J = 0.96, 8.07 Hz, 1H), 8.26-8.25 (m, 1H), 7.77 (td, J = 1.86, 7.80 Hz, 1H), 7.29-7.23 (m, 2H), 4.71 (s, 2H), 4.28 (br. s, 1H).

EXAMPLE 4



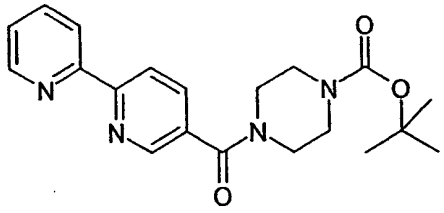
3-Aminomethyl-2,2'-bipyridine. 3-hydroxymethyl-2,2'-bipyridine (0.19mmol, 36 mg) was dissolved in dry THF (5 ml), with triethylamine (0.3 ml) at ambient temperature before PS-tosyl chloride (200 mg) was added. The resulting suspension was shaken for 3h before the resin was removed by filtration and washed sequentially with dimethylformamide (2 x 5 ml), tetrahydrofuran (2 x 5 ml) and dichloromethane (2 x 5 ml). To the resin was added dry dichloromethane (5 ml) then through this suspension was passed a stream of ammonia gas for a period of 10 min. The vessel was sealed and the suspension shaken for 72 h. The solids were removed by filtration and the residue was with dichloromethane (2 x 5 ml). The combined organics were concentrated *in vacuo* to give the *amine* which was used without further manipulation. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.68 (m, 2H), 8.38 (m, 2H), 7.84 (m, 2H), 7.32 (m, 1H), 3.98 (s, 2H).

EXAMPLE 5



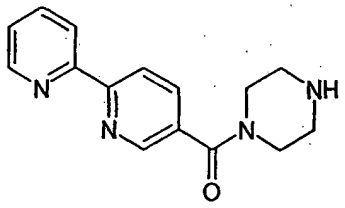
1-[2,2']Bipyridinyl-5-ylmethyl-pyrrole-2,5-dione. The amine from Example 4 (0.2mmol, 37 mg) was dissolved in dichloromethane (5 ml) at ambient temperature before the sequential addition of maleic anhydride (0.2mmol, 20 mg), PS-carbodiimide (200 mg) and hydroxybenzotriazole monohydrate (0.2mmol, 27 mg). The suspension was stirred for 36 h before the addition of PS-isocyanate (200 mg). After stirring for a further 2 h, the solids were removed by filtration and the residue washed with dichloromethane (2 x 5ml). The combined organics were concentrated *in vacuo*. Purification by column chromatography on alumina (10% MeOH/DCM). Yield 7 %. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.65 (m, 2H), 8.36 (m, 2H), 7.82 (m, 2H), 7.32 (m, 1H), 6.75 (m, 2H), 4.75 (m, 2H).

EXAMPLE 6



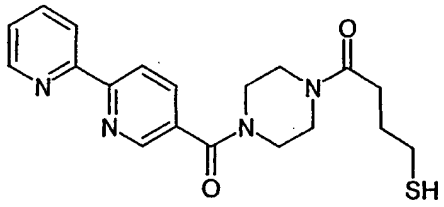
4-([2,2']Bipyridinyl-5-carbonyl)-piperazine-1-carboxylic acid tert-butyl ester. To a dry mixture of *N*-butoxycarbonyl piperazine (5mmol, 1.1 g), 2,2'-bipyridyl-4-carboxylic acid (5 mmol, 1.0 g), EDC (6.5mmol, 1.25 g) and hydroxybenzotriazole monohydrate (6.0 mmol, 0.81 g) was added dry dichloromethane (50 ml). The mixture was stirred at ambient temperature for 16 h before being washed with a saturated solution of sodium bicarbonate (10 ml), water (10 ml), brine (10 ml), dried over sodium sulphate and condensed *in vacuo*. The product was used without further manipulation. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.72 (m, 2H), 8.50 (d, J = 8.0 Hz, 1H), 8.43 (d, J = 8.0 Hz, 1H), 7.88 (m, 2H), 7.36 (m, 1H), 3.90-3.25 (m, 8H), 1.52 (s, 9H).

EXAMPLE 7



[2,2']Bipyridinyl-5-yl-piperazin-1-yl-methanone. The product from Example 6 (0.19 mmol, 73 mg) was dissolved in dichloromethane (5 ml) at ambient temperature. Trifluoroacetic acid (1 ml) was added and stirring continued for 1 h. The reaction mixture was washed with water (2 x 10 ml) and the combined aqueous basified to pH 10 before being extracted with dichloromethane (2 x 10 ml). The combined organics were washed with brine, dried over sodium sulphate and concentrated *in vacuo* to give the *amine*. Yield: 47 %. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.72 (m, 2H), 8.48 (d, J = 8.1 Hz, 1H), 8.42 (d, J = 8.1 Hz, 1H), 7.85 (m, 2H), 7.35 (dd, J = 7.9, 4.9 Hz, 1H), 3.90-3.40 (br m, 4H), 3.50- 2.80 (br m, 4H).

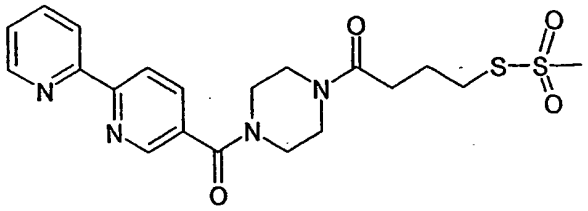
EXAMPLE 8



1-[4-([2,2']Bipyridinyl-5-carbonyl)-piperazin-1-yl]-4-mercapto-butan-1-one. The amine from Example 7 (1.56 mmol, 401 mg) was dissolved in dry dimethylamine (15 ml) with triethylamine (1.5 ml) at ambient temperature. The solution was deoxygenated by refluxing under a nitrogen atmosphere, before γ-thiolactone (1.87mmol, 0.16 ml) was added and the solution was heated to 120 °C for 16 h. A second portion of γ-thiolactone (1.87mmol, 0.16 ml) was added and heated continued for a further 1 h. The reaction mixture was allowed to cool to ambient temperature before dichloromethane (100 ml) was added and the solution extracted with hydrochloric acid (3 x 20 ml, 1M). The combined aqueous were basified to pH 10 and re-extracted with dichloromethane (3 x 20 ml). The combined organics were washed with water (20 ml), brine (20 ml) dried over sodium sulphate and concentrated *in vacuo*. Purification by column chromatography (40 % [10 %

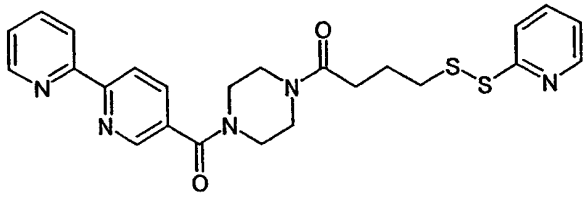
Et_3N in EtOAC]/petrol) yielded the *thiol*. Yield: 25 %, ^1H NMR $CDCl_3$, 300 MHz): δ 8.74 (m, 2H), 8.51 (d, J = 7.9 Hz, 1H), 8.44 (d, J = 7.9 Hz, 1H), 7.90 (m, 2H), 7.37 (m, 1H), 4.00-3.40 (br. m, 8H), 2.65 (m, 2H), 2.52 (m, 2H) 1.98 (m, 2H).

EXAMPLE 9



Methanethiosulfonic acid S-{4-[4-([2,2']bipyridinyl-5-carbonyl)-piperazin-1-yl]-4-oxo-butyl} ester. The thiol from Example 8 (0.135mmol, 50 mg) was dissolved in dichloromethane (0.5 ml) under nitrogen atmosphere before methanesulphonyl chloride (0.149mmol, 11.5 μl) was added. The solution was stirred for 2 h before a further portion of methanesulphonyl chloride (0.149mmol, 11.5 μl) was added and the solution stirred for a further 16 h. The volatiles were removed *in vacuo*. ^1H NMR DMSO, 300 MHz): δ 8.80 (m, 2H), 8.52 (m, 2H), 8.18 (m, 2H), 7.65 (m, 1H), 6.10-5.20 (br. m, 8H), 3.59 (s, 3H), 3.25-3.15 (m, 6H).

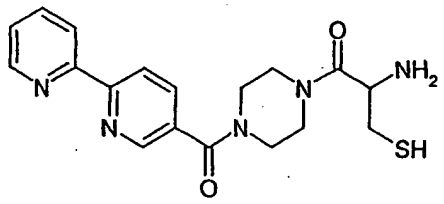
EXAMPLE 10



1-[4-([2,2']Bipyridinyl-5-carbonyl)-piperazin-1-yl]-4-(pyridin-2-yldisulfanyl)-butan-1-one. The thiol from Example 8 (0.043mmol, 16 mg) and 2,2'-dipyridyldisulfide (0.086mmol, 19 mg) were stirred together in a mixture of ethanol (1 ml) and acetic acid (33 μl) for 24 h. The volatiles were removed *in vacuo*. Purification by column chromatography on alumina (60 % EtOAC/petrol) yielded the *disulfide*. Yield: 38 %. ^1H NMR $CDCl_3$, 300 MHz): δ 8.73 (m, 2H), 8.51 (d, J = 8.3 Hz, 1H), 8.46 (d, J = 2.7 Hz, 1H), 8.44 (d, J = 8.3 Hz, 1H), 7.88

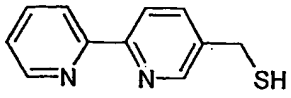
(m, 2H), 7.68 (m, 2H), 7.36 (dd, J = 4.9, 4.9 Hz, 1H), 7.11 (m, 1H), 3.90-3.45 (br. m, 8H), 2.90 (t, J = 6.8 Hz, 2H), 2.53 (t, J = 6.4 Hz, 2H), 2.10 (app. q, J = 6.8, 6.4 Hz, 2H).

EXAMPLE 11



2-Amino-1-[4-([2,2']bipyridinyl-5-carbonyl)-piperazin-1-yl]-3-mercapto-propan-1-one. Amine from Example 7 (0.4 mmol, 100 mg), *N*-Boc-*S*-Tr-*L*-cysteine (0.44 mmol, 204 mg), EDC (□□mmol, 100 mg) and HOBT (0.48 mmol, 65 mg) were dissolved in dichloromethane (4 ml) at ambient temperature and stirred for 16 h. The reaction mixture was diluted with dichloromethane (10 ml) before a saturated solution of sodium bicarbonate (5 ml) was added until the pH of the solution reached 8. Extract with dichloromethane (3 x 10 ml). The combined extracts were dried over sodium sulphate and concentrated *in vacuo*. The crude material was dissolved in dichloromethane (5 ml) before trifluoroacetic acid (0.4 ml) and triisopropylsilane (0.2 ml) was added. A saturated solution of sodium bicarbonate was added until the solution pH was 8. Extract with dichloromethane (3 x 5 ml), the combined organics were dried over sodium sulphate and concentrate *in vacuo*. Yield: 17 %, ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.72 (m, 1H), 8.49 (m, 1H), 7.88 (m, 1H), 7.45 (m, 2H), 7.30 (m, 2H), 3.90-2.90 (m, 11H).

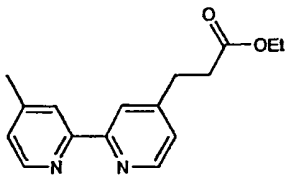
EXAMPLE 12



5-Thiomethyl-2,2'-bipyridine. 5-Hydroxymethyl-2,2'-bipyridine (2.0 g, 10.7 mmol) was added thionyl chloride (40 ml, 0.55 mol), and the reaction was heated to 70 °C for 2 h. The reaction was allowed to reach room temperature, and concentrated to give 5-chloromethyl-2,2'-bipyridine dihydrochloride. The crude 5-chloromethyl-2,2'-bipyridine dihydrochloride (137 mg, 0.67 mmol) in MeOH (3 ml) was added thiourea (140 mg, 1.84

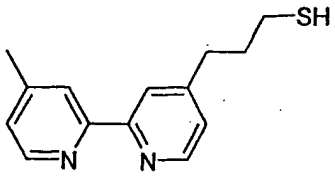
mmol), and the reaction mixture was heated to reflux. After 2.5 h 4M NaOH (2 ml) was added, and the reaction was heated to reflux for another 3 h. The reaction mixture was added NH_4Cl until pH 7, and extracted with CH_2Cl_2 (3x). The extract was concentrated and purified by flash chromatography (basic Al_2O_3; EtOAc:heptane, 1:2). Yield: 55 mg (39 %). ^1H NMR $CDCl_3$, 300 MHz); δ 8.70 (m, 1H), 8.64 (m, 1H), 8.43 (m, 1H), 8.40 (m, 1H), 7.80-7-90 (m, 2H), 7.33 (ddd, J = 8.7, 3.6, 1.1 Hz, 1H), 3.82 (d, J = 7.7 Hz, 2H), 1.84 (t, J = 7.7 Hz, 1H). GC/MS: m/z = 202 [= M]$^+$

EXAMPLE 13



3-(4'-Methyl-[2,2']bipyridinyl-4-yl)-propionic acid ethyl ester. 4,4'-Dimethyl-2,2'-bipyridyl (2.5 g, 13.5 mmol) was dissolved in dry THF (20 ml) under a nitrogen atmosphere, in a flame-dried flask, equipped with a stirrer. The solution was cooled to –78 °C, and a solution of LDA (10 ml, 16.8 mmol) was added. The reaction mixture was allowed to warm to room temperature for 1,5 hours. This solution was cannulated into a solution of ethyl 2-bromoacetate (2.3 ml, 20 mmol) in dry THF (15 ml) at –78 °C, placed in a flame-dried flask under a nitrogen atmosphere, equipped with a stirrer. The reaction mixture was allowed to reach room temperature slowly over night, and quenched by addition of a saturated aqueous solution of sodium bicarbonate. Extractive work-up using ethyl acetate, drying and evaporation, gave the crude product. Purified by column chromatography (Silica; DCM:MeOH:NH_4OH 95:5:0.5). Yield: 1.86 g (51 %). ^1H NMR ($CDCl_3$, 300 MHz) δ 1.17 (t, J = 7.16 Hz, 3H), 2.40 (s, 3H), 2.66 (t, J = 7.63 Hz, 2H), 2.99 (t, J = 7.63 Hz, 2H), 4.07 (q, J = 7.16 Hz, 2H), 7.13 (m, 2H), 8.22 (s, 1H), 8.28 (s, 1H), 8.49 (d, J = 5.08 Hz), 8.52 (d, J = 5.09 Hz, 1H).

EXAMPLE 14

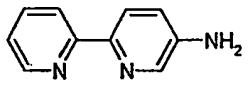


4-(3-Mercaptopropyl)-4'-methyl-2,2'-bipyridine. A solution of 3-(4'-methyl-[2,2']bipyridinyl-4-yl)-propionic acid ethyl ester (from Example 13) (0.98 g, 3.63 mmol) in dry THF (35 ml) at -78 °C was added dropwise DIBAL (1.0 M in THF, 8.0 ml, 8.0 mmol). The reaction was slowly allowed to reach room temperature (over 12 h), and then quenched with MeOH (5 ml). Rochelle's salt (sat. aq., 15 ml) was added, and the mixture was stirred for 3 h. The mixture was extracted with EtOAc. The extract was washed with brine and concentrated. The crude product was purified by flash chromatography (SiO_2, [EtOAc w/10% TEA]:heptane, 1:1, then 2:1) to give 523 mg (63 %) of 4-(3-hydroxypropyl)-3'-methyl-5,5'-bipyridine. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.69-8.52 (m, 2H), 8.28-8.20 (m, 2H), 7.20-7.10 (m, 2H), 3.75-3.65 (m, 2H), 2.85-2.77 (m, 2H9, 2.45 (s, 3H), 2.05-1.90 (m, 2H).

A solution of 4-(3-hydroxypropyl)-4'-methyl-5,5'-bipyridine (114 mg, 0.50 mmol) in CH_2Cl_2 (12 ml) was added Et_3N (1.38 ml, 1.00 mmol). The reaction was cooled to 0 °C and tolsyl chloride (143 mg, 0.75 mmol) was added. The reaction was allowed to reach room temperature, and stirred for 12 h. Concentration and purification by flash chromatography (SiO_2, [EtOAc w/10 % Et_3N]:heptane, 1:2, then 1:1) gave 116 mg (61 %) of 4-(3-tosyloxypropyl)-4'-methyl-5,5'-bipyridine. ^{1}H NMR $CDCl_3$, 300 MHz): δ 8.56-8.50 (m, 2H), 8.26 (s, 1H), 8.24 (s, 1H), 7.78 (d, J = 8.3 Hz, 2H), 7.32 (d, J = 8.5 Hz, 2H), 7.20-7.15 (m, 1H), 7.08-7.04 (m, 1H), 4.06 (t, J = 6.0 Hz, 2H), 2.76 (t, J = 8.3 Hz, 2H), 2.46 (s, 3H), 2.43 (s, 3H), 2.12-2.01 (m, 2H).

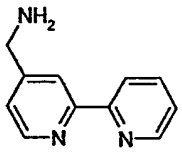
A solution of thiourea (30 mg, 0.37 mmol) in water (0.8 ml) was added a solution of 4-(3-tosyloxypropyl)-4'-methyl-5,5'-bipyridine (93 mg, 0.24 mmol) in ethanol (0.8 ml), and the reaction was heated to reflux. After 9 h 1.0 M NaOH (0.7 ml) was added, and the reaction was heated to reflux for 2 h. The reaction was cooled to room temperature, added 1.0 M HCl until pH 7, and extracted with EtOAc. The extract was washed with brine and concentrated. The residue was purified by flash chromatography (Al_2O_3, EtOAc: heptane, 1:3) Yield: 22.4 mg (38 %) of the title compound. ^{1}H NMR (MeOH-d_4, 300 MHz): δ 8.52 (d, J = 5.1 Hz, 1H), 8.48 (d, J = 5.1 Hz, 1H), 8.13-8.08 (m, 2H), 7.33-7.28 (m, 2H), 2.87 (t, J = 7.2 Hz, 2H), 2.55 (t, J = 7.2 Hz, 2H), 2.46 (s, 3H), 2.00 (p, J = 7.2 Hz, 2H).

EXAMPLE 15



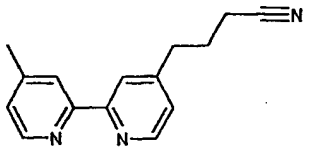
5-Amino-2,2´-bipyridine. 5-Nitro-2,2'-bipyridine (0.641 mol, 129 mg) was dissolved in MeOH/THF (5 ml+5 ml). To the solution was added Pd/C (5 %, 50 mg) and the reaction mixture was set under an H_2-atmosphere and stirred for 24 h at room temperature. The reaction mixture was filtered through Celite, and the filtrate was evaporated *in vacuo*. The residue was purified by column chromatography (neutral Al_2O_3, 5 % EtOH in DCM), to yield the desired product. Yield: quantitative. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.63-8.61 (m, 1H), 8.27-8.15 (m, 3H), 7.76 (td, J = 1.9, 7.8 Hz, 1H), 7.22 (m, 1H), 7.09 (dd, J = 2.8, 8.4 Hz, 1H), 3.88 (br. s, 2H).

EXAMPLE 16



4-(Aminomethyl)-2,2´-bipyridine. 2,2'-Bipyridine-4-carboxamide (15.0 mmol, 3.0g) was placed in an oven dried 100 ml round bottomed flask equipped with stirrer magnet. Borane tetrahydrofurane complex (30 mmol, 30 ml, 1M solution in THF) was slowly added, and the content of the flask was stirred for 15 h. The reaction mixture was quenched with saturated NH_4Cl (aq). The mixture was made basic (pH = 9) with 1M NaOH. The resulting mixture was stirred for 1h at room temperature after which the organic phase was separated, and the aqueous phase was extracted twice with EtOAc (2x30 ml). The combined organic phases were dried over $MgSO_4$, and filtered through a sintered glass funnel. The solvent was removed by evaporation *in vacuo*.

EXAMPLE 17



4-(3-Cyanopropyl)-4'-metyl-2,2'-bipyridyl: 4,4'-Dimethyl-2,2'-bipyridyl (5.0 g, 27 mmol) was dissolved in dry THF (50 ml) under a nitrogen atmosphere, in a flame-dried flask, equipped with a stirrer. The solution was cooled to –78 °C, and a solution of LDA (20 ml, 33 mmol) was added. The reaction mixture was allowed to warm to room temperature for 1,5 hours. This solution was cannulated into a solution of 3-bromopropionitrile (3.4 ml, 40 mmol) in dry THF (20 ml) at –78 °C, placed in a flame-dried flask under a nitrogen atmosphere, equipped with a stirrer. The reaction mixture was allowed to reach room temperature slowly over night, and quenched by addition of a saturated aqueous solution of sodium bicarbonate. Extractive work-up using ethyl acetate, drying and evaporation, gave the crude product of major components being starting material and expected product. The crude product was purified by column chromatography (Alumina; EtOAc:Heptane 1:2). Yield: 2.1 g (33 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.02 (m, 2H), 2.32 (t, J = 7.07 Hz, 2H), 2.39 (s, 3H), 2.81 (t, J = 7.63 Hz, 2H), 7.10 (m, 2H), 8.20 (s, 1H), 8.24 (s, 1H), 8.47 (d, J = 5.09 Hz, 1H), 8.54 (d, J = 5.09 Hz, 1H).

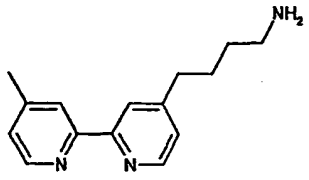
5-(3-Cyanopropyl)-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 0.67 g (52 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.04 (m, 2H), 2.39 (t, J = 6.97 Hz, 2H), 2.42 (s, 3H), 2.86 (t, J = 7.54 Hz, 2H), 7.68 (d, J = 8.26 Hz, 2H), 8.32 (d, J = 7.91 Hz, 1H), 8.40 (d, J = 8.29 Hz, 1H), 8.53 (s, 2H).

4-(2-Cyanoethyl)-4'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 1.17 g (19 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.48 (s, 3H), 2.77 (t, J = 7.35 Hz, 2H), 3.08 (t, J = 7.44 Hz, 2H), 7.22 (m, 2H), 8.30 (s, 1H), 8.37 (s, 1H), 8.56 (d, J = 4.90 Hz, 1H), 8.66 (d, J = 4.98 Hz, 1H).

5-(2-Cyanoethyl)-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 27 mg (8 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.39 (s, 3H), 2.68 (t, J = 7.25 Hz, 2H), 3.02 (t, J = 7.35 Hz, 2H), 7.59-7.73 (m, 2H), 8.28 (m, 1H), 8.36 (m, 1H), 8.51 (m, 1H), 8.56 (m, 1H).

5-Cyanomethyl-5'-metyl-2,2'-bipyridyl: Same procedure as described above. Yield: 51 mg (15 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.39 (s, 3H), 3.81 (s, 2H), 7.63 (m, 1H), 7.79 (m, 1H), 8.28 (d, J = 8.10 Hz, 1H), 8.40 (d, J = 8.10 Hz, 1H), 8.51 (m, 1H), 8.60 (m, 1H).

EXAMPLE 18

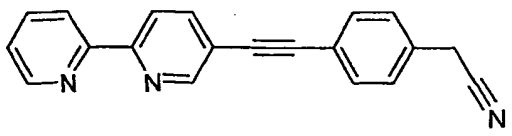


4-(4-Aminobutyl)-4'-methyl-2,2'-bipyridyl: 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridyl (125 mg, ca. 0.5 mmol) was dissolved in 96 % ethanol (5 ml) and catalytic amount of Raney nickel was added. The reaction was stirred over night under 1 atmosphere of hydrogen. Evaporated and purified by chromatography (alumina, DCM:MeOH:NH_4OH 95:5:0.5). Yield: 70 mg (58 %). ^1H NMR ($CDCl_3$, 300 MHz) δ 1.49 (m, 2H), 1.68 (m, 2H), 2.36 (s, 3H), 2.64 (t, J = 7.72 Hz, 2H), 2.68 (s, 2H), 2.70 (t, J = 7.07 Hz, 2H), 7.05 (m, 2H), 8.15 (m, 2H), 8.46 (dd, J = 0.47, 4.99Hz, 1H), 8.48 (dd, J = 0.66, 5.00Hz, 1H).

5-(4-Aminobutyl)-5'-methyl-2,2'-bipyridyl: Same procedure as described above. Yield: 181.4 (44 %). ^1H NMR ($CDCl_3$, 300 MHz) δ 1.52 (m, 2H), 1.70 (m, 2H), 1.80 (s, 2H), 2.38 (s, 3H), 2.68 (t, J = 7.54 Hz, 2H), 2.74 (t, J = 7.06 Hz, 2H); 7.59 (m, 1H), 7.62 (m, 1H), 8.24 (d, J = 6.03 Hz, 1H), 8.26 (d, J = 8.10 Hz, 1H), 8.48 (s, 1H), 8.49 (s, 1H).

4-(3-Aminopropyl)-4'-methyl-2,2'-bipyridyl: Same procedure as described above. Yield: 190 mg (50 %). ^1H NMR (DMSO-d_6, 300 MHz) δ 1.80 (m, 2H), 2.41 (s, 3H), 2.68 (t, J = 7.16 Hz, 2H), 2.74 (t, J = 7.82 Hz, 2H), 4.48 (s, broad, 2H), 7.28 (m, 2H), 8.23 (s, 1H), 8.25 (s, 1H), 8.53 (d, J = 5.08 Hz, 1H), 8.56 (d, J = 5.08 Hz, 1H).

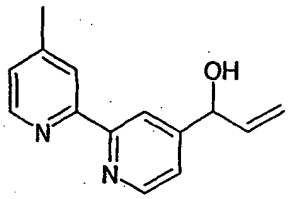
EXAMPLE 19



(4-[2,2']Bipyridinyl-5-ylethynyl-phenyl)-acetonitrile. 4-(2-(2'-Pyridyl)pyridyl)acetylene (0.6 g, 2.0 mmol), iodophenylacetonitril (0.54 g, 2.2 mmol), copperiodide (38 mg, 0.2 mmol),

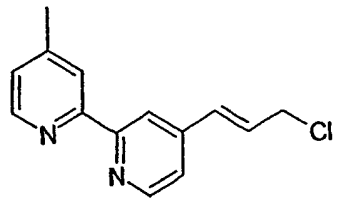
tetrakis(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol) and triethylamine (2.8 ml, 20 mmol) in DMF (10 ml) was stirred at R.T. under nitrogen for 24 hours. The reaction was reduced *in vacuo*, and water and ethylacetate added. The organic layer was dried, reduced *in vacuo* and purified on a silica column, using ethylacetate/ether (1:1) as eluent. Recrystalised in ethylacetate. Yield: 30 mg (5 %). ^{1}H NMR($CDCl_3$, 300 MHz) δ 3.82 (s, 1H), 7.35 (m, 1H), 7.38 (d, J = 8.1 Hz, 2H), 7.61 (d, J = 8.1 Hz, 2H), 7.85 (br t, J = 7.7 Hz, 1 H), 7.97 (dd, J = 8.3 Hz, J = 2.1 Hz, 1H), 8.45 (d, J = 8.1 Hz, 2H), 8.72 (d, J = 4.9 Hz, 1H), 8.85 (m, 1H).

EXAMPLE 20



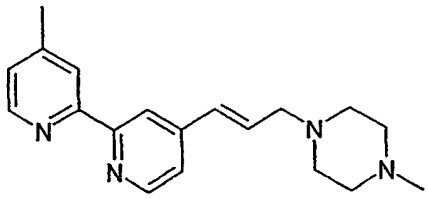
1-(4'-Methyl-[2,2']bipyridinyl-4-yl)-prop-2-en-1-ol. 4-Formyl-4'-methyl-2,2'-bipyridine (10.1 mmol, 2.0 g) was dissolved in dry tetrahydrofuran (100 ml) at -20 °C before vinyl magnesium bromide (12.0 mmol, 1M, 12.0 ml) was added dropwise. The reaction mixture was stirred for 2h before a saturated aqueous solution of ammonium chloride (50 ml) was added. The resulting mixture was extracted with ethyl acetate (3 x 100 ml); the organics were combined, washed with brine (100 ml), dried and evaporated. Purification by column chromatography (40 % [10 % Et_3N in EtOAC]/petrol) yielded the *alcohol* as an orange solid. Yield 63 %, ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.56 (d, 1H, J = 5.1 Hz), 8.48 (d, 1H, J = 5.1 Hz), 8.34 (m, 1H), 8.17 (m, 1H), 7.29 (m, 1H), 7.08 (m, 1H), 5.98 (ddd, J = 16.0, 10.0, 3.9 Hz, 1H), 5.34 (dt, J = 16.0, 1.3 Hz, 1H), 5.23 (br. d, J = 16.0 Hz, 1H), 5.17 (dt, J = 10.0, 1.3 Hz, 1H), 2.39 (s, 3H).

EXAMPLE 21



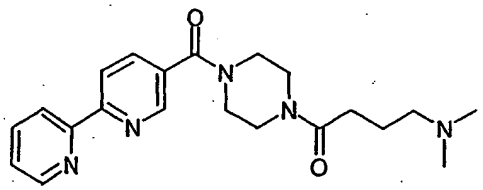
4'-(3-Chloro-propenyl)-4-methyl-[2,2']bipyridinyl. The alcohol from Example 20 (6.3 mmol, 1.5 g) was dissolved in dry dichloromethane (30 ml) and stirred at 0 °C before thionyl chloride (30 ml) was added in one portion. The reaction was stirred until TLC showed consumption of all starting material. The reaction mixture was allowed to warm to room temperature before careful addition of water (50 ml) and sodium bicarbonate (50 ml). The mixture was then extracted with dichloromethane (3 x 100 ml); the combined organics were dried over silica and then filtered through a plug of celite before being concentrated *in vacuo* to yield a yellow oil which was used without purification.

EXAMPLE 22



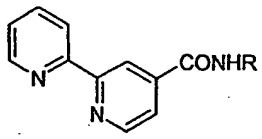
4-Methyl-4'-[3-(4-methyl-piperazin-1-yl)-propenyl]-2,2'-bipyridine. The chloride from Example 21 (1.32 mmol, 0.342 g) was dissolved in dry dichloromethane (25 ml) at ambient temperature. 1-Methylpiperazine (13.2 mmol, 1.13 g) was added and the solution was stirred overnight. The reaction mixture was extracted with hydrochloric acid (3 x 20 ml, 1M). The combined aqueous were washed with dichloromethane (10 ml), basified to pH 10 and extracted with dichloromethane (3 x 50 ml). The combined organics were washed with brine (50 ml) dried over sodium sulphate and then concentrated *in vacuo*. chromatography (10 % MeOH/DCM) yielded the *amine* as a mixture of geometric isomers. Yield: 66 %, ^{1}H NMR($CDCl_3$, 300 MHz) (major isomer reported): δ 8.53 (d, J = 5.1 Hz, 1H), 8.46 (d, J = 5.1 Hz, 1H), 8.31 (s, 1H), 8.16 (s, 1H), 7.18 (m, 1H), 7.06 (m, 1H), 6.65-6.48 (m, 2H), 3.14 (m, 2H), 2.60-2.40 (br. s, 8H), 2.38 (br. s, 3H), 2.24 (br. s, 3H).

EXAMPLE 23



1-[4-([2,2']Bipyridinyl-5-carbonyl)-piperazin-1-yl]-4-dimethylamino-butan-1-one. A screw-top vial was charged with PS-carbodiimide resin (200 mg) followed by a solution of γ-dimethylaminobutyric acid (0.15 mmol, 25 mg) in dichloromethane (1 ml). The suspension was stirred gently for 5 min. before the addition of a solution of the amine from Example 7 (0.1 mmol, 25 mg) in dichloromethane (1 ml). Stirring continued for 16 h before the addition of PS-trisamine (200 mg) and further stirring for 2 h. The solids were removed by filtration and the residue washed with dichloromethane (10 ml). The combined organics were dried *in vacuo* to give the tertiary amine. Yield: 41 mg (99 %). 1H NMR ($CDCl_3$, 300 MHz): δ 8.70 (m, 2H), 8.45 (d, J = 8.1 Hz, 1H), 8.40 (d, J = 8.1 Hz, 1H), 7.85 (m, 2H), 7.38 (dd, J = 4.9, 4.7 Hz, 1H), 3.95- 3.45 (m, 8H), 3.35 (t, J = 7.2 Hz, 2H), 2.80 (s, 6H), 2.68 (t, J = 6.5 Hz, 2H), 2.54 (app. q, J = 7.0 Hz, 2H).

EXAMPLE 24

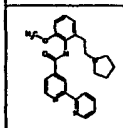
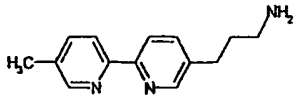
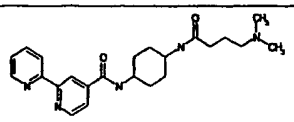
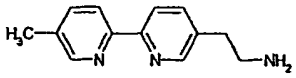
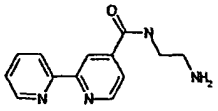
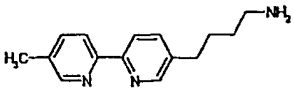
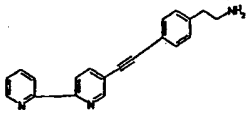
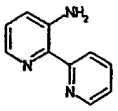
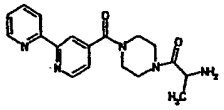
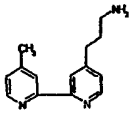
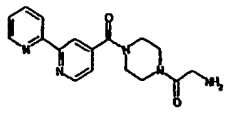
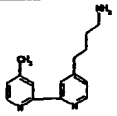
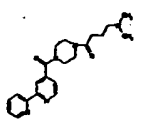
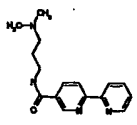


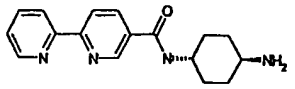
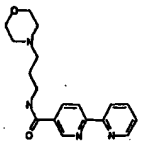
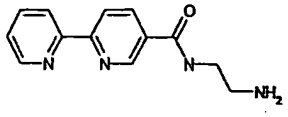
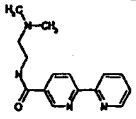
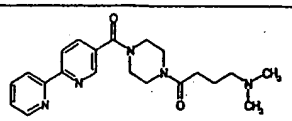
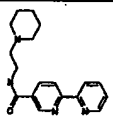
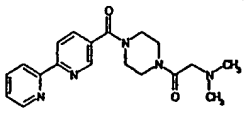
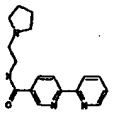
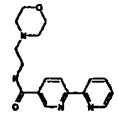
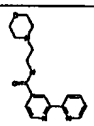
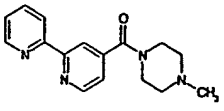
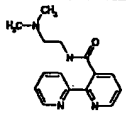
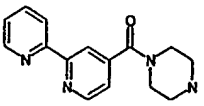
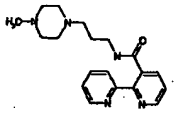
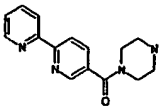
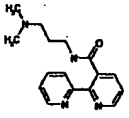
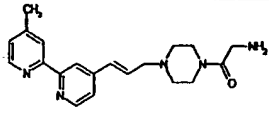
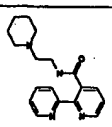
Sodium bipyridinecaboxylate (0.4 mmol, 88.9 mg) was dissolved in 4 ml DMF/CH_2Cl_2 (1:1). Acetic acid (0.4 mmol), coupling reagent (HBTU, TFFH or EDC) (0.4 mmol, 151.7 mg), amine (0.4 mmol) and triethyl amine (0.4 mmol) were added and shaken for 18h at room temperature. The reaction mixture was then quenched with aqueous NaOH (2 ml, 2M), and extracted into 10 ml CH_2Cl_2. The solvent was evaporated at room temperature. Purification was performed either by HPLC (acetonitrile/water in a gradient of acetonitrile in 85 %→ 0 % at a flow rate of 10 ml/min. Column type : YMC-ODS 250x10 mm) or column chromatography on neutral alumina (eluent: acetone/heptane with acetone in a

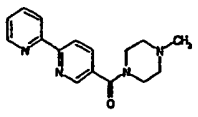
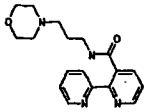
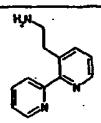
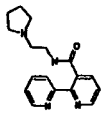
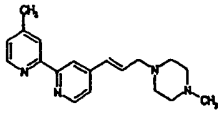
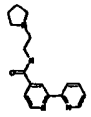
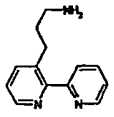
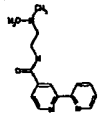
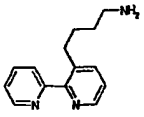
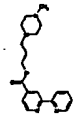
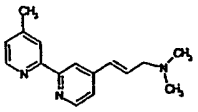
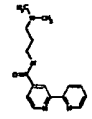
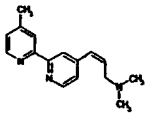
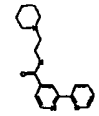
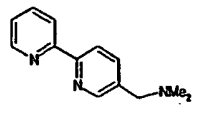
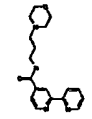
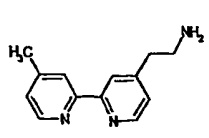
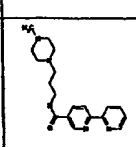
gradient of 20 %→40 %). The desired amides were identified by LC/MS, using 5mM NH_4OAc as the mobile phase, and ES as the ionisation technique.

In analogous manners 2,2´-bipyridyl amines are synthesised according to Table 1, giving an representative example for a "C"-type library.

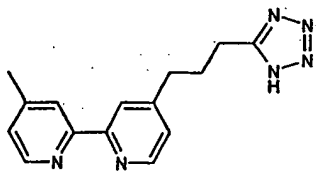
Table 1 2,2´-Bipyridyl amines in a "C"-type library.

Structure	Method	Structure	Method
	Ex 24		Ex 18
	Ex 23		Ex 18
	Ex 7		Ex 18
	Ex 18		Ex 15
	Ex 23		Ex 18
	Ex 23		Ex 18
	Ex 23		Ex 24

	Ex 7		Ex 24
	Ex 7		Ex 24
	Ex 23		Ex 24
	Ex 23		Ex 24
	Ex 24		Ex 24
	Ex 6		Ex 24
	Ex 7		Ex 24
	Ex 7		Ex 24
	Ex 23		Ex 24

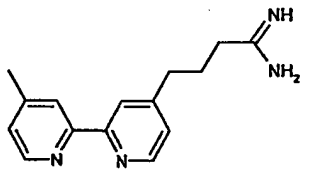
	Ex 6		Ex 24
	Ex 18		Ex 24
	Ex 22		Ex 24
	Ex 18		Ex 24
	Ex 18		Ex 24
	Ex 22		Ex 24
	Ex 22		Ex 24
	Ex 22		Ex 24
	Ex 18		Ex 24

EXAMPLE 25



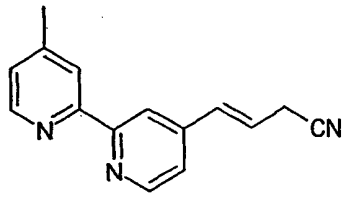
4'-Methyl-4-[3-(1H-tetrazol-5-yl)-propyl]-2,2'-bipyridine. 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridyl (0.72 g, 3 mmol) was dissolved in dry toluene (10 ml), followed by addition of sodium azide (0.6 g, 9 mmol) and triethylammonium chloride (1.25 g, 9 mmol). The reaction was heated to 100 °C for 18 hours. After cooling, a small amount of water is added, the phases separated, and the aqueous phase acidified with hydrochloric acid. The crude product precipitated as a red oil, which is purified on a column (silica, EtOAc:MeOH 1:2). Yield: 0.6 g (71 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.13 (m, 2H), 2.41(s, 3H), 2.69 (t, J = 7.35 Hz, 2H), 2.92 (t, J = 7.45 Hz, 2H), 5.79 (s, broad, 1H)), 7.08 (m, 1H), 7.14 (m, 1H), 8.02 (s, 1H), 8.07 (s, 1H), 8.46 (d, J = 5.27 Hz, 1H), 8.48 (d, J = 5.09 Hz, 1H).

EXAMPLE 26



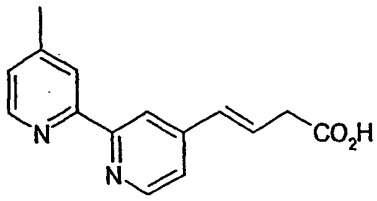
4-(4-Butyramidine)-4'-methyl-2,2'-bipyridine. Dry NH_4Cl (0.17g, 3mmol) in dry toluene (3 ml) was stirred at an ice-bath under nitrogen, and trimethylaluminium (1.6 ml, 2.0M, 3.2 mmol) added slowly. The mixture was allowed to attain room temperature. 4-(3-Cyanopropyl)-4'-methyl-2,2'-bipyridine (0.25 g, 1 mmol) was added, and the reaction is heated to 90 °C for 3 days. Alumina (9 g) was suspended in chloroform (40 ml), and the reaction mixture poured into it, followed by methanol (50 ml), and the reaction mixture was stirred for 0.5 hours. The slurry was filtered and concentrated *in vacuo*. Extractive work-up in DCM and aqueous $NaHCO_3$. Purification on alumina column (heptane:ethylacetate:ethanol (2:2:1)). Yield: 0.05 g (18 %). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.85 (p, J = 7.7 Hz, 2H), 2.10 (t, J = 7.7 Hz, 2H), 2.42 (s, 3H), 2.68 (t, J = 7.7 Hz, 2H), 6.74 (br. s, 1H), 7.28 (m, 2H), 8.23 (m, 2H), 8.55 (m, 2H).

EXAMPLE 27



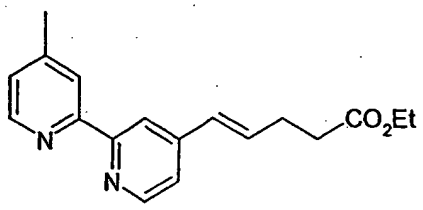
4-(4'-Methyl-[2,2']bipyridinyl-4-yl)-but-3-enenitrile. The chloride from Example 21 (3.5 mmol, 0.868g) was dissolved in dry ethanol (40 ml) at ambient temperature. Potassium cyanide (4.3 mmol, 0.209g) dissolved in water (3.5 ml) was added in one portion and the resulting solution was heated to 78 °C for 15 h. The solvent was then removed *in vacuo* before the crude product was subjected to column chromatography (40 % [10 % Et_3N in EtOAC]/petrol) yielded the cyanide as an orange solid. Yield: 18 %, ^1H NMR ($CDCl_3$, 300 MHz): δ 8.65 (d, J = 5.1 Hz, 1H), 8.55 (d, J = 5.1 Hz, 1H), 8.43 (s, 1H), 8.24 (s, 1H), 7.29 (dd, J = 4.9, 0.8 Hz, 1H), 7.16 (dd, J = 4.9, 0.8 Hz, 1H), 6.80-6.55 (m, 2H), 3.78 (d, J = 7.2 Hz, 2H), 2.45 (s, 3H).

EXAMPLE 28



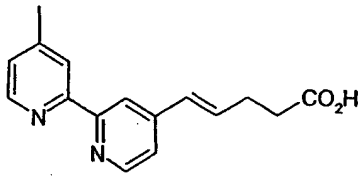
4-(4'-Methyl-[2,2']bipyridinyl-4-yl)-but-3-enoic acid. The cyanide from Example 27 (0.48 mmol, 0.112g) was taken up in sodium hydroxide solution (20 mmol, 4M, 5 ml) and stirred for 15h at reflux. The solvent was then remove *in vacuo* and the product purified on chromatotron (EtOAc,/petrol gradient). Yield 3.2 %, ^1H NMR ($CDCl_3$, 300 MHz): δ 8.62 (d, J = 5.1 Hz, 1H), 8.55 (d, J = 5.1 Hz, 1H), 8.39 (s, 1H), 8.25 (s, 1H), 7.27 (m, 1H), 7.16 (m, 2H), 6.71 (m, 1H), 4.41 (m, 2H), 2.46 (s, 3H).

EXAMPLE 29



5-(4'-Methyl-[2,2']bipyridinyl-4-yl)-pent-4-enoic acid ethyl ester. The alcohol from Example 20 (0.64 mmol, 0.144g) was dissolved in triethyl orthoacetate (2 ml), and toluene (8 ml) under a nitrogen atmosphere. Acetic acid (20 μl) was added and the resulting solution was heated to 120 °C for 3 h. After cooling to ambient temperature, a saturated solution of sodium carbonate (10 ml) was added and the mixture extracted with ethyl acetate (3 x 20 ml). The combined organics were washed with brine (50 ml) before the addition of petrol (120 ml). The organic solution was passed through a plug of silica and the filtrate reduced *in vacuo*. Purification by column chromatography (20 % [10 % Et_3N in EtOAC]/petrol) yielded the *ester* as an pale yellow solid. Yield: 40 %.

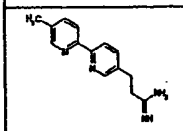
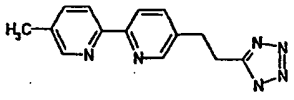
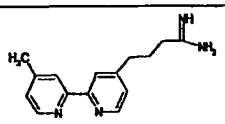
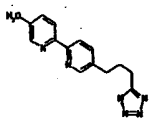
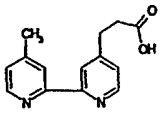
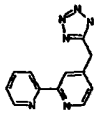
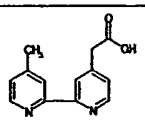
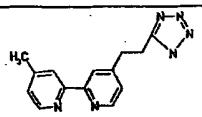
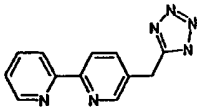
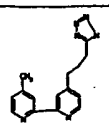
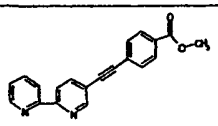
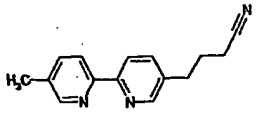
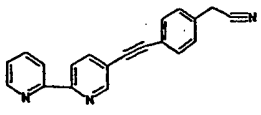
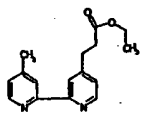
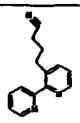
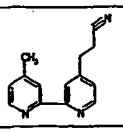
EXAMPLE 30

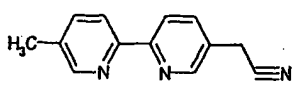
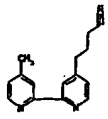
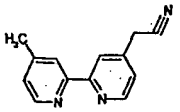
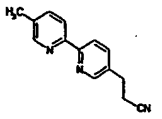


5-(4'-Methyl-[2,2']bipyridinyl-4-yl)-pent-4-enoic acid. Ethyl ester from Example 29 (0.045 mmol, 0.020g) was taken up in a mixture of THF (0.5 ml), ethanol (0.5 ml) and water (0.1 ml) at ambient temperature before potassium carbonate (0.045 mmol, 0.063 g) was added and the resulting suspension stirred overnight. Barium hydroxide (0.1 g) was added and the suspension stirred for a further 7 h before the pH of the mixture was adjusted to 5 and the mixture extracted with ethyl acetate (3 x 5 ml). The combined organics were dried and reduced *in vacuo*. Yield: 50 %, ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.60 (m, 2H), 8.33 (s, 1H), 8.24 (s, 1H), 7.21 (dd, J = 6.1, 4.9 Hz, 1H), 6.66 (m, 1H), 6.51 (d, J = 15.8 Hz, 1H), 2.62 (m, 4H), 2.46 (s, 3H).

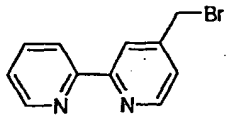
In analogous manners the following functional groups were introduced to the 2,2'-bipyridine scaffold, shown in Table 2, giving representative examples for various kinds of "C"-type libraries.

Table 2 Various functionalized 2,2'-bipyridines as part of various "C"-type libraries.

Structure	Method	Structure	Method
	Ex 26		Ex 25
	Ex 26		Ex 25
	Ex 30		Ex 25
	Ex 30		Ex 25
	Ex 25		Ex 25
	Ex 19		Ex 17
	Ex 19		Ex 13
	Ex 17		Ex 17

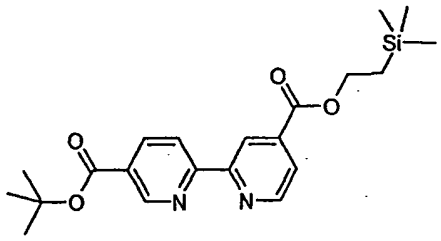
	Ex 17		Ex 17
	Ex 17		Ex 17

EXAMPLE 31



4-(Bromomethyl)-2,2´-bipyridine. 4-(Hydroxymethyl)-2,2´-bipyridine (5.37 mmol, 1.0 g) was dissolved in DMF (15 ml). PBr_3 (5.37 mmol, 0.5 ml) was added dropwise at room temperature under inert atmosphere. The reaction mixture was stirred at room temperature for 15 h. Water (50 ml) was added as the reaction vessel was cooled on an ice-bath. Ethyl acetate (100 ml) was added, and sat. $NaHCO_3$ (100 ml) was added. The organic layer was separated and the aqueous phase was extracted with ethyl acetate (2x50 ml). The combined organic layers were dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. Column chromatography of the crude material (DCM/MeOH, 100/10) yielded the pure bromo methyl compound. Yield. 60 %. ^1H NMR ($CDCl_3$, 300 MHz): δ 8.69-8.66 (m, 2H), 8.44-8.41 (m, 2H), 7.84 (td, J= 1.86, 7.5 Hz, 1H), 7.36-7.31 (m, 2H), 4.49 (s, 2H).

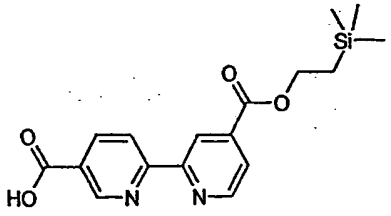
EXAMPLE 32



4-(2-(trimethylsilyl)-ethylcarboxylate)-5´-(tert-butylcarboxylate)-2,2´-bipyridine. 6-Chloro-*tert*-butylnicotinate (12.7 mmol, 2.7g) was dissolved in dry *m*-xylene (150 ml) whereupon $Me_3SnSnMe_3$ (15.26 mmol, 5.0 g) was added together with $PdCl_2(PPh_3)_2$ (1.5 mmol, 1.0g). The reaction solution was heated to 130C under an N_2 atmosphere for 4h. 2-Chloro-(2-(trimethylsilyl)ethyl)-*iso*-nicotinate (15.26 mmol, 3.9g) was added and stirring was continued at 130C for 16h. The reaction mixture was allowed to cool to ambient temperature whereafter the solvent was evaporated *in vacuo*. The residue was taken up in DCM, and purified by column chromatography using DCM as the eluent. Yield: 62 %. ^1H NMR ($CDCl_3$, 300 MHz): δ 9.29-9.25 (m, 1H), 9.05-9.02 (m, 1H), 8.88-8.84 (m, 1H), 8.56-8.51 (m, 1H), 8.42-8.36 (m, 1H), 7.96-7.92 (m, 1H), 4.53-4.48 (m, 2H), 1.65 (s, 9H), 1.24-1.18 (m, 2H), 0.12 (s, 9H).

By the same method the corresponding 2,2´-bipyridyl-esters, -nitriles, -aldehydes, -protected amines and protected alcohols be synthesised in all possible combinations, and positions on the 2,2´-bipyridyl scaffold (*i.e.* AA´-, AC-libraries).

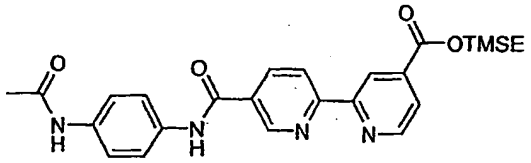
EXAMPLE 33



4-(2-(trimethylsilyl)-ethylcarboxylate)-5´-(carboxyacid)-2,2´-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5´-(*tert*-butylcarboxylate)-2,2´-bipyridine (2.5 mmol, 1.0g) was dissolved in dry 1,4-dioxane (15 ml). Triethylamine (3.75 mmol, 0.523 ml) was added and TMSOTf (3.75 mmol, 0.679 ml) was added droppwise. Upon completion of addition the reaction solution was heated to 100C for 2h. Stirring was thereafter continued for 3h at 21C. Water

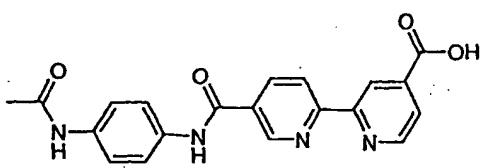
was then carefully added and the formed precipitate was collected by filtration and the solid residue was washed several times with water and allowed to dry at room temperature for 24h. Yield: 71 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.21-9.18 (m, 1H), 8.94-8.90 (m, 1H), 8.87-8.83 (m, 1H), 8.54-8.52 (m, 1H), 8.45-8.42 (m, 1H), 7.93-7.90 (m, 1H), 4.49-4.43 (m, 2H), 1.18-1.15 (m, 2H), 0.08 (s, 9H).

EXAMPLE 34



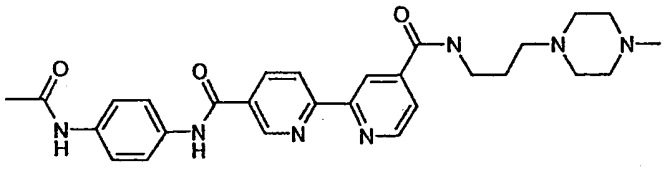
4-(2-(trimethylsilyl)-ethylcarboxylate)-5′-(4-(acetanilido)carboxamide)-2,2′-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5′-(carboxyacid)-2,2′-bipyridine (1.45 mmol, 0.5g) was dissolved in DCM/DMF (5 ml / 5 ml). HBTU (1.74 mmol, 0.66g) was added and the mixture was stirred for 2h at room temperature. 4-Aminoacetanilide (1.74 mmol, 0.26g) was added in one portion, and stirring was continued at room temperature for another 16h. Water was added, and the reaction mixture was extracted with DCM. The combined organic phases were washed once with water and finally with brine prior to drying over $MgSO_4$, and evaporation *in vacuo.* Purification was made by column chromatography on neutral alumina using DCM/ ethanol (95:5) as eluent. Yield: 72 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.48 (s, 1H), 9.94 (s, 1H), 9.25-9.22 (m, 1H), 8.98-8.93 (m, 1H), 8.90-8.88 (m, 1H), 8.57-8.54 (m, 1H), 8.50-8.46 (m, 1H), 7.94-7.92 (m, 1H), 7.70 (d, J = 8.85 Hz, 2H), 7.58 (d, J = 9.03 Hz, 2H), 4.51-4.46 (m, 2H), 2.04 (s, 3H), 1.19-1.14 (m, 2H), 0.09 (s, 9H).

EXAMPLE 35



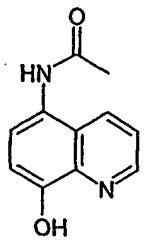
4-(carboxyacid)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine. 4-(2-(Trimethylsilyl)-ethylcarboxylate)-5´-(4-(acetanilido)-carboxamide)-2,2´-bipyridine (0.042 mmol, 20 mg) was dissolved in THF (5 ml). To the solution was added TBAF (0.126 mmol, 126□l, 1M solution in THF). The solution was stirred at room temperature for 15h, whereupon it was made acidic with diluted HCl (1M solution) to pH = 3.5. The formed precipitate was collected by filtration and rinsed with several portions of water, and thereafter dried at room temperature for 24h. Yield: 67 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 13.80 (br. s, 1H), 10.47 (s, 1H), 9.92 (s, 1H), 9.24-9.23 (m, 1H), 8.95-8.84 (m, 2H), 8.57-8.54 (m, 1H), 8.48 (dd, J = 2.07, 7.95 Hz, 1H), 7.93 (dd, J = 1.68, 7.80 Hz, 1H), 7.70 (d, J = 8.46 Hz, 2H), 7.57 (d, J = 9.03 Hz, 2H), 2.04 (s, 3H).

EXAMPLE 36



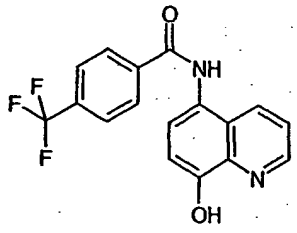
4-(3´´-(N-methylpiperazine)-propyl)carboxamide)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine. 4-(Carboxyacid)-5´-(4-(acetanilido)carboxamide)-2,2´-bipyridine (0.04 mmol, 15 mg) was dissolved in DCM/DMF (1:1, 5 ml), whereupon HBTU (0.05 mmol , 189 mg) was added in one portion. A few drops of triethylamine was added and the resulting mixture was stirred at room temperature for 3 h. 3-(*N*`-Methylpiperazine)-propyl amine (0.06 mmol , 9.4 mg) was added, and the reaction solution was stirred at room temperature over night. Water was added and the the organic layer was separated. The aqueous phase was extracted with DCM. The combined organic layers were washed with water, brine and finally sat. $CaCl_2$. Drying over $MgSO_4$, and evaporation *in vacuo* yielded a yellow viscous oil. Purification was made by column chromatography on neutral alumina using DCM/ ethanol (95:5) as eluent. Yield: 63 %. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.38 (s, 1H), 9.81 (s, 1H), 9.21-9.19 (m, 1H), 8.91-8.82 (m, 2H), 8.58-8.55 (m, 1H), 8.46 (dd, J = 1.97, 7.89 Hz, 1H), 7.90 (dd, J = 1.73, 7.81 Hz, 1H), 7.69 (d, J = 8.51 Hz, 2H), 7.53 (d, J = 9.10 Hz, 2H), 3.45-3.30 (m, 2H), 2.60-2.30 (m, 10H), 2.18 (s, 3H), 2.04 (s, 3H), 1.18-1.16 (m, 2H).

EXAMPLE 37



N-(8-Hydroxy-quinolin-5-yl)-acetamide. 5-Amino-8-hydroxyquinoline (1 mmol, 0.233g) was stirred in ether at ambient temperature before acetic anhydride (10 mmol, 1ml), followed by sodium acetate (10 mmol, 1.36g) was added. The resulting mixture was heated to 40 °C for 16 h before being diluted with ether (100ml) poured onto a saturated solution of ammonium chloride (50 ml). The organics were separated and washed with sodium bicarbonate (50 ml), water (3 x 50 ml), brine (50 ml), dried over sodium sulphate and concentrated *in vacuo*. Purification by column chromatography (30 % EtOAc/petrol). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.11 (dd, J = 8.9, 1.7 Hz, 1H), 9.4 (dd, J = 4.0, 1.5 Hz, 1H), 8.46 (d, J = 8.4 Hz, 1H), 7.71 (dd, J = 8.8, 4.1 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 2.56 (s, 3H).

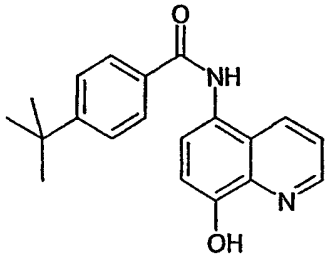
EXAMPLE 38



N-(8-Hydroxy-quinolin-5-yl)-4-trifluoromethyl-benzamide. 5-Amino-8-hydroxyquinoline (0.15mmol, 25 mg) was dissolved in dry dichloromethane (5 ml) before the sequential addition of dimethyl formamide (0.2 ml), *N,N,*-dimethylaminopyridine (1 crystal), PS-carbodiimide (750mg) and 1-hydroxybenzotriazole monohydrate (0.6mmol, 81 mg). The suspension was stirred for 72 h before the solids were removed by filtration and the resulting filtrate diluted with dichloromethane (20 ml), washed with sodium bicarbonate (2 x 20 ml), brine (20 ml), dried over sodium sulphate and concentrated *in vacuo*. The

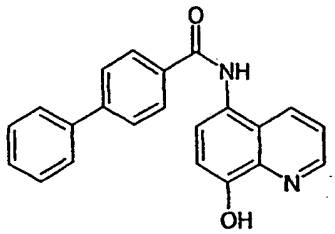
residue was then taken up in dichloromethane (50 ml), with methanol (10 ml) and water (1 ml). To this mixture was added lithium hydroxide (30mmol, 720 mg). The suspension was stirred for 16 h before the solids were removed by filtration and the resulting filtrate washed with sodium bicarbonate (20 ml), water (20 ml) and brine (20 ml), dried over sodium sulphate and purified by direct filtration through a plug of alumina. The alumina was washed with dichloromethane (100 ml) before the product was eluted with ethyl acetate. The volatiles were removed *in vacuo*. GC-MS: m/z = 332 (= M^+).

EXAMPLE 39



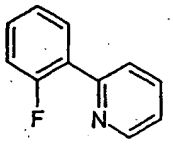
4-tert-Butyl-N-(8-hydroxy-quinolin-5-yl)-benzamide. To a suspension of 5-amino-8.hydroxyquinoline dihydrochloride (1.0mmol, 0.23 g) and dimethylaminopyridine (3 crystals) in dichloromethane (10 ml) at ambient temperature was added 4-*tert*-butylbenzoyl chloride (3.0 mmol, 0.59 ml). Stirring continued for 10 min before triethylamine (10mmol, 2.8 ml) was added in one portion. The solution was allowed to stir overnight before all volatiles were removed *in vacuo* and the residue purified directly by column chromatography (10 % EtOAc / hexane). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.13 (dd, J = 9.0, 1.7 Hz, 1H), 8.99 (dd, J = 4.1, 1.7 Hz, 1H), 8.52 (d, J = 8.5 Hz, 1H), 8.27 (dt, J = 8.5, 1.7 Hz, 2H), 7.69 (m, 2H), 7.61 (m, 2H), 1.43 (s, 9H).

In similar fashion the following compound was made:



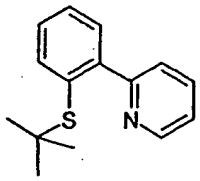
^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.14 (dd, J = 8.9, 1.5 Hz, 1H), 9.03 (dd, J = 4.0, 1.4 Hz, 1H), 8.57 (d, J = 8.5 Hz, 1H), 8.43 (app d, J = 8.1 Hz, 2H), 7.84 (app. d, J = 8.3 Hz, 2H), 7.70 (m, 4H), 7.50 (m, 4H).

EXAMPLE 40



2-(2-Pyridyl)fluorobenzene: 2-Fluorophenylboronic acid (3.0 g, 21.4 mmol) was dissolved in DME (40 ml). 2-Bromopyridine (1.64 ml, 17.2 mmol) was added followed by 2M K_2CO_3 (20 ml). The mixture was degassed by bubbling nitrogen gas through for 34 min. Bis-(triphenylphosphine)palladium chloride (1.2 g, 1.72 mmol) was added and the mixture was heated to 80°C over night. The mixture was cooled to room temperature and filtered through celite. Extraction with H_2O (200 ml) and EtOAc (200 ml), drying the organic phase over $MgSO_4$, filter and evaporation gave the crude product. Purification by column chromatography (SiO_2, DCM: 10 % NH_4OH in MeOH 10:0.05). Yield: 2.4 g (80 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.18 (dt, J = 8.1, 1.13 Hz, 1H), 7.28 (m, 2H), 7.40 (m, 1H), 7.79 (m, 2H), 8.00 (dt, J = 7.72, 1.88 Hz, 1H), 8.75 (dt, J = 4.52, 1.32 Hz, 1H). LC-MS: m/z = 174 (=M+1)

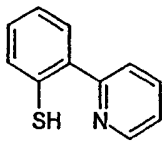
EXAMPLE 41



S-tert-Butyl-2-(2-pyridyl)thiophenol: DMF (10 ml) was degassed for 1hour and 10 minutes. Sodium hydride (60 % dispersion in mineral oil) (231 mg, 5.77 mmol) and 2-methyl-2-propanethiol (715 µl, 5.77 mmol) was added. The mixture was stirred for 7 minutes at room temperature. 2-(2-Pyridyl)fluorobenzene (500 mg, 2.89 mmol) was added, and the

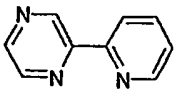
mixture was heated to 120 °C for 3 days. The mixture was cooled to room temperature. H_2O (50 ml) was added and the mixture was extracted with EtOAc (70 ml). The organic phase was washed with H_2O (50 ml), dried over $MgSO_4$, filtered and evaporated. Yield: ~100 %, ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.04 (s, 9H), 7.25 (m, 1H), 7.39 (dt, J = 7.54, 1.51 Hz, 1H), 7.49 (dt, J = 7.54, 1.51 Hz, 1H), 7.69 (m, 4H), 8.70 (m, 1H). LC-MS: m/z = 244 (=M+1)

EXAMPLE 42



2-(2-Pyridyl)thiophenol: S-*tert*-Butyl-2-(2-pyridyl)thiophenol (200 mg, 0.82 mmol) was dissolved in 37 % HCl (4 ml) and the mixture was heated to 110 °C over night. The mixture was cooled to room temperature. H_2O (10 ml) was added and the mixture was extracted with EtOAc (20 ml). pH of the aqueous phase was adjusted to 7 and the mixture was extracted with EtOAc (50 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (SiO2, EtOAc:Heptane 1:1). Yield: 66.2 mg (43 %), ^{1}H NMR (CDCl3, 300 MHz) δ 7.30 (m, 3H), 7.52 (m, 1H), 7.62 (m, 1H), 7.80 (m, 2H), 8.74 (m, 1H).

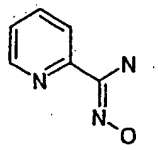
EXAMPLE 43



2-(2-Pyridyl)pyrazine. 2-Chloropyrazine (100 mg, 0.87 mmol) was dissolved in m-xylen (2 ml), 2-tri-*n*-butylstannylpyridin (354 mg, 0.96 mmol) was added followed by bis-(triphenylphosphine)palladium chloride (1.2 mg, 0.0017 mmol). The mixture was heated to 130 °C over night under nitrogen. The mixture was allowed to cool to room temperature. The crude mixture was purified by column chromatography (SiO2; EtOAc:Heptane 1:1). The product was dissolved in EtOAc (25 ml) and washed with aqueous HCl (pH ~ 3) (2 x 30 ml). The aqueous phase was adjusted to pH 8 with $NaHCO_3$ and extracted with EtOAc

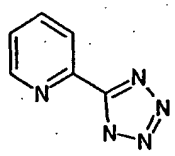
(2 x 20 ml). The organic phase was dried over Na_2SO_4, filtered and evaporated. Yield: 53 mg (38 %). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.39 (m, 1H), 7.87 (dt, J = 7.91, 1.69 Hz, 1H),8.38 (m, 1H),8.62 (m, 2H),8.74 (m, 1H), 9.66 (s, 1H).

EXAMPLE 44



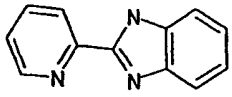
N-Hydroxy-pyridine-2-carboxamidine. Sodium (0.53 g 23 mmol) was dissolved in MeOH (15 ml), hydroxylamine hydrochloride (1.53 g 22 mmol) dissolved in MeOH (15 ml) was added, and stirred in ice bath for 1 hour. After filtration the solution was added 2-cyanopyridin (1.93 ml, 20mmol),and stirred at R.T. over night. The reaction mixture was reduced *in vacuo*. After cooling on ice the product precipitate. Filtered and washed with diethyl ether. Yield: 2.1 g (73 %). ^1H NMR ($CDCl_3$, 300 MHz) δ 5.75 (br s, 2H), 7.34 (ddd J = 1.32 Hz, J = 4.9 Hz, J = 7.53 Hz, 1H), 7.73 (dt, J = 1.88 Hz, J = 7.54 Hz, 1H), 8.94 (dt, J = 1.13 Hz, J = 7.92Hz, 1H), 8.18 (br s, 1H), 8.58 (ddd, J = 0.95 Hz, J = 1.88 Hz, J = 4.9 Hz, 1H).

EXAMPLE 45



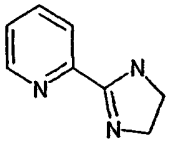
2-(5-Tetrazolyl)pyridyine. This chelator was prepared from the corresponding cyano-compound according to Example 25.

EXAMPLE 46



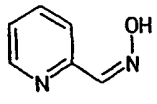
2-Pyridin-2-yl-1H-benzoimidazole. Picolinic acid (2.5 g, 20.3 mmol) was added THF (25 ml) and heated to reflux. Carbonyl diimidazole (3.6 g, 22.3 mmol) was added in portions, and the reaction was heated for 3 hours. After cooling to R.T., 1,2-phenylenediamine (2.2 g, 20.3 mmol) was added, and the reaction was stirred for 1 hour at R.T. Evaporated and dissolved in EtOAc, washed with water, dried and evaporated. The formed crystals were washed with diethyl ether and dried. Yield: 0.8 g.

EXAMPLE 47



2-(4,5-Dihydro-1H-imidazol-2-yl)-pyridine. Picolinic acid (2.5 g, 20.3 mmol) was added THF (25 ml) and heated to reflux. Carbonyl diimidazole (3.6 g, 22.3 mmol) was added in portions, and the reaction was heated for 3 hours. After cooling to R.T., 1,2-ethylenediamine (1.4 ml, 20.3 mmol) was added, and a colorless precipitation was formed. The reaction was stirred for 1 hour at R.T. The solid was filtered off, washed with THF and dried. Yield: 1.3 g.

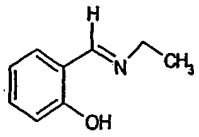
EXAMPLE 48



Pyridine-2-carbaldehyde oxime. 2-Pyridylcarbaldehyde (0.5 g, 4.7 mmol) and hydroxylamine hydrochloride (0.65 g, 9.4 mmol) was dissolved in ethanol (30 ml) followed by pyridine (0.76 ml, 9.43 mmol). The reaction was heated to reflux for 2 hours and 20 minutes. After cooling, the reaction was evaporated, the crude redissolved in EtOAc,

washed with water, dried and evaporated, to give a colourless crystalline solid. Yield: 0.46 g (80 %).

EXAMPLE 49



2-Ethyliminomethyl-phenol. Salicylaldehyde (4.4 ml, 40.9 mmol) was dissolved in dry toluene (100 ml), and ethylamine (g) was bubbled through (3 x 5 minutes). Left at R.T. over night. Still starting material. The reaction was heated to 65 °C over night. Evaporated and distilled.

The following examples presented encompass naturally occurring as well as specifically engineered metal-ion binding sites in a number of different proteins representing several different classes of membrane proteins: 7TM proteins (examples being various G-protein coupled receptors), and 12TM proteins (example - the dopamine transporter) as well as an example comprising a soluble protein, Factor VIIa, the active form of the FVII protease.

The examples are chosen with the intent of illustrating the sequential and rational process through which small organic compounds, the metal-ion chelators, may be identified as ligands and subsequently optimized with respect to the affinity by which they recognize the protein targets.

Overall, the examples serve to illustrate how the activity of potential drug targets may be affected through interaction with small metal-ion chelators and importantly how the present technology provides the opportunity to aim the active drug candidates towards functionally significant domains of the target. Throughout this section, 'the affinity' of the metal-ion chelator complexes refers to the ability of the complex to displace the binding of a radioligand and the potency of the metal-ion chelator complexes refers to the ability of the substances to activate or inactivate the drug targets.

I. Identification and binding Of Metal-Ions and Metal-Ion Complexes To Various Drug Targets With Natural Metal-Ion Sites

The examples compiled in this section illustrate how metal-ion binding sites may be identified in the native forms of various drug targets, and how these sites may be addressed by metal-ions in complex with certain chelators, as observed either through an effect on the binding affinity of a radioactive ligand or through a direct effect on activation or inactivation of the target.

Example I.1 – Identification of a naturally occurring metal-ion chelator binding-site in the 7TM leukotriene LTB4 receptor

The present example illustrates how the presence of a previously unnoticed, naturally occurring metal-ion binding site within a transmembrane segment of a 7TM receptor may be predicted through analysis of the nucleotide sequence of the gene coding for the protein and how it can subsequently be experimentally identified. Briefly, molecular models of 7TM receptors can be built based on the deduced amino acid sequence and identification of the seven transmembrane segments (eg.Unger at al. (1997) Nature 389: 203-206). In these molecular models, illustrated in the helical wheel diagram shown in Fig. 5, potential metal-ion sites can be identified by the presence of metal-ion binding residues, for example histidine, cysteine, or aspartate residues located in suitable relative positions, for example in an *i* and *i + 4* arrangement (i.e. with three residues in between) on a helical face within the so-called main ligand-binding crevice of the receptor between TM-II, III, IV, V, VI, and VII (Schwartz et al, (1996) Trends Pharmacol. Sci. 17: 213-216).

Methods – The leukotriene LTB4 receptor cDNA was cloned by PCR from a leukocyte cDNA library, built into an eukaryotic expression vector and introduced into COS-7 cells by a standard calcium phosphate transfection method. One day after transfection the cells were transferred and seeded in multi-well plates for assay. The number of cells plated per well was chosen so as to obtain 5 to 10% binding of the radioligand added. Two days after transfection the cells were assayed for the presence of [^{3}H]-LTB4 binding activity. Radioligand was bound in a buffer composed of 50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1 % BSA, 100 mg/ml Bacitracin and displaced in a dose dependent manner by unlabelled LTB4 ligand. The assay was performed in duplicate for 3 hours at 4 °C, and stopped by washing twice in buffer. Cell associated, receptor bound radioligand was determined by the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid). The concentration of radioligand in the assay corresponds to a final concentration of 45 pM. The metal-ion chelating complex, 2,2'-bipyridine was added in a two-fold molar excess in order to ensure that no free metal-ion was present.

Results and discussion - As shown in the helical wheel diagram of the leukotriene LTB4 receptor (Fig. 5C), two Cys residues are located on the face of TM-III pointing inwards, i.e. towards the main ligand-binding pocket of the receptor (positions III:04, Cys^{93} and III:08, Cys^{97}). Theoretically these residues could constitute a metal-ion binding site. The actual presence of a naturally occurring metal-ion binding site in the leukotriene LTB4 receptor is demonstrated by the fact, that binding of the radioligand, [^{3}H]-LTB4 to the receptor expressed in COS-7 cells could be displaced by Cu(II), IC_{50} = 70 µM (Fig.5A). In agreement with the fact that the proposed metal-ion site is located in the main ligand-binding pocket of the receptor, i.e. with amble space towards the center of the receptor, the complex between the metal-ion and the chelator, 2,2'-bipyridine bound equally well as the free metal-ion, i.e. the 2,2'-bipyridine did neither impair nor improve the binding affinity (Fig.5A). As shown in Fig. 5B, Ala-substitution of Cys^{93} severely impaired the effect of the metal-ion chelator complex on LTB4 binding. Ala-substitution of Cys^{97} also clearly impaired the effect of the metal-ion complex. The combined substitution of both Cys residues totally eliminated the metal-ion chelator effect (Fig. 5B) demonstrating that these two residues on the central face of TM-III are involved in the binding of the metal-ion chelator complex. Thus, the two residues represent a naturally occurring intra-helical 'bis-Cys-site', which can be addressed with for example Cu(II) in complex with bipyridine..

Example I.2 – Identification of naturally occurring metal-ion binding site in the 7TM galanin receptor-1

The galanin receptor-1 (Gal-R1) provides yet another example of a 7TM receptor that contains naturally, endogenous metal-ion binding sites. Interestingly, this receptor has the potential of shifting its "chelation-mode" between two distinct chelation configurations.

Methods - The galanin receptor-1 cDNA was introduced into COS-7 cells by the standard calcium phosphate transfection method. The cells were transferred and seeded in multi-well plates for assay one day following the transfection and the number of cells plated per well was adjusted for each individual (wild type and mutant) construct aiming at the binding of 5 to 10% of the radioligand present in the assay. Two days post-transfection the cells were assayed for the presence of [^{125}I]-Galanin binding activity. Radioligand was bound in buffer composed of 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 100 mg/ml Bacitracin and displaced in a dose dependent manner by unlabelled ligand. The assay was performed in duplicate for 3 hours at 4 °C, and terminated by washing twice in ice-cold buffer, followed by the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid). The concentration of radioligand in the assay corresponds to a final concentration of 20

pM. Mutations were created in the cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59).

Results and discussion - Binding of [^{125}I]-galanin to the Gal-R1 expressed in COS-7 cells is displaced by Zn(II) with an IC_{50} of 40 micromolar. From analysis of a molecular model of the Gal-R1 putative chelating residues in the receptor was identified and subjected to mutational analysis, Fig 6G. Importantly, by mutating either His267, His263 or His264 to Gln, Ala, Ala respectively, the apparent affinity for Zn(II) was significantly decreased suggesting that these residue are crucial for the observed high affinity of the wildtype Gal-R1 for Zn(II), Fig 62A, D, E. Interestingly, the double mutant [His263Q;His264Q] was virtually unresponsive to Zn(II), Fig 6.F. In contrast, mutating another residue in proximity of His263, His264 and His 267, in the molecular model, His112 to Ala, had no effect on the apparent affinity of Zn(II) suggesting that this residue does not participate in the chelation of Zn(II), Fig. 6.C. From analysis of a large number of naturally occurring metal-ion sites in proteins, it is well know for those skilled in the art, that an acidic residue if often located in proximity of the chelating histidines, where it by hydrogen bonding to one of the chelating histidines fine-tunes the histidine for chelation of the metal-ion. Consistent with this, mutating Glu271 to Gln, located one helical turn above the important His267, slightly decreased the apparent affinity for Zn(II), Fig. 6B. A model consistent with these data, is depicted in Fig 6H, I. In binding mode I, the metal-ion is chelated primarily by residues His263 and His267, optimally spaced in an i, i+4 configuration in the helix. Upon mutation of His263, a new binding mode is observed, where the metal-ion is chelated by residues His264 and His267. The opposite is observed upon mutation of His264, when the binding mode shift solely to binding mode I. Alternatively, in the wildtype receptor, all three histidines 263, 264 and 267 chelates the metal-ion. However, in that model, the configuration of the histidines is not optimal for chelation and since the observed Zn(II) affinity is more consistent with a bidentate site, we favor a bidentate model.

These experiments demonstrate that naturally occurring intra-helical metal-ion sites in 7TM receptors can be identified, dissected by molecular analysis and addressed by a metal-ions or metal-ions in complex with chelators.

Example I.3 – Identification of naturally occurring metal-ion chelator binding site in the 12TM dopamine transporter.

In the literature, a naturally occurring allosteric metal-ion binding site has been demonstrated in the dopamine transporter, a membrane protein having supposedly 12 transmembrane spanning segments, 12TM (Norregaard et al EMBO J. 17: 4266-4273 (1998); Loland et al., JBC, 274, 36928-34, (1999)). Here Zn(II) binds in a two-component fashion to a tridentate metal-ion site composed of residues His^{193}, His^{375}, and Glu^{396} and thereby blocks dopamine transport. This effect of Zn(II) can be eliminated by mutational exchange of any of the three residues with a non-chelating residue.

Methods - The dopamine transporter cDNA was introduced into COS-7 by the standard calcium phosphate transfection method. Two days post-transfection the cells were assayed for [^{3}H]-Dopamine uptake activity. The uptake assays was performed in 25 mM Hepes pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid and 5 mM D-glucose and in the presence of various concentrations of unlabelled dopamine as indicated in the figures. The assay was performed in triplicate at 37°C for 10 minutes, and terminated by washing with buffer twice and the addition of lysis buffer (48% urea, 2% NP-40 in 3M acetic acid).

Results and discussion - As shown in Fig. 7A, 2,2'-bipyridine in complex with Zn(II) inhibits the transport of [^{3}H]dopamine by the dopamine transporter, transiently expressed in COS-7 cells, in a two component fashion, i.e. with IC_{50} values of 0.16 and 20 μM, corresponding to a slightly higher potency than the free metal-ion, which similarly acts in a two component fashion, i.e. with IC_{50} values of 2.2 and 338 ?M,. Importantly, the chelator bipyridine had no effect on the dopamine transport without being on complex with the metal-ion (Fig.7A). That the metal-ion chelator complex acts through the same site as the free metal-ion was demonstrated by the mutational exchange of residue His^{193} (Fig. 7B). Dopamine transport could be inhibited also by a structurally distinct class of metal-ion chelators, exemplified by 2-pyridylamidoxime,O-acetyl (compound 210), which like 2,2'-bipyridine does not affect dopamine transport by itself, but blocks dopamine transport with a potency approx. 10-fold higher than free Zn(II) and interestingly acts in a mono-component fashion (Fig. 7C). This effect of the metal-ion chelator complex was eliminated by mutational substitution of His^{193} known to be involved in metal-ion binding (Fig. 7C). This substitution is known not to affect the transport of catecholamine (Norregaard et al (1998) EMBO J. 17: 4266-4273) indicating that the effect of the metal-ion chelator complexes is mediated through the binding to a site (i.e. the endogenous metal-ion site), which is different from the catecholamine binding site. Thus, the metal-ion chelator complexes act as blockers of transport through a novel allosteric molecular mechanism and could therefore serve as lead compounds in the development of a new type of

transport blockers. It should be noted that the affinity of, for example 2-pyridylamidoxime,O-acetyl (compound 210) corresponds to even a very good lead compound found by simple screening.

The experiments presented in this section demonstrate that metal-ion chelator complexes of very different chemical structures can act as allosteric blockers of function - in these cases of either 7TM receptors or 12TM transporter proteins - through binding to naturally occurring metal-ion sites. Furthermore, it is shown that these compounds can bind with affinities similar to that of lead compounds found by conventional drug screening techniques. Thus, these metal-ion chelators can function as lead compounds in a chemical optimization process to obtain high affinity compounds acting as drug candidates.

II. Binding Of Metal-Ion Complexes In Engineered Metal-Ion Sites In Various Potential Drug Targets

Natural metal-ion sites are only found in a subset of potential drug targets. However, through mutagenesis it is possible to introduce metal-ion binding sites in proteins by introduction of metal-ion binding residues such as His, Cys, or Asp. The examples in the present section demonstrate how metal-ion complexes can bind to and affect the function of proteins after mutational engineering of metal-ion sites into the proteins.

Example II.1 – Binding of various metal-ion complexes to a library of inter-helical metal-ion sites engineered into the tachykinin NK1 receptor.

This example illustrates that different epitopes of a target protein - here a NK1 receptor - can be addressed by metal-ion chelator complexes, i.e. potential lead compounds for antagonists, after systematic mutational engineering of metal-ion sites into these different epitopes. Previously, a series of metal-ion sites have been built into the tachykinin NK1 receptor to probe helix-helix interactions, i.e. providing distance constraints in molecular models of the receptor (Elling et al. (1995) Nature 374: 74-77, Elling et al. (1996) EMBO J. 15: 6213-6219; Holst et al. (2000) Mol.Pharmacol. 58: 263-270). Here, such metal-ion sites are used as anchor points for potential lead compounds - i.e. metal-ion chelator complexes - for the development of receptor antagonists with different molecular mechanisms of actions.

Methods – The tachykinin NK1 receptor cDNA was expressed in COS-7 cells. Two days after transfection whole cells were assayed with respect to binding of radioactively labeled substance P ([^{125}I]-Bolton Hunter labeled Substance P), in displacement with substance P, $ZnCl_2$, $CuCl_2$ or various chelator complexes thereof present in a three fold molar ratio with respect to the metal-ion concentration. The zinc(cyclam) complex was prepared by co-incubation at 60 °C for one hour followed by overnight incubation at 37 °C. The assay was typically performed in 12 or 24 well plates. On the day of assay, the cells were washed with binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 0.1 % BSA, 0.1 % and Bacitracin (100 mg/ml). Unlabelled competitor ligand and radioligand (20,000 cpm – approximately 20 pM) was added to the cells in binding buffer and incubation continued for 3 hours at 4 °C. The assay was terminated by washing of the cells and lysis. The assay was performed in duplicate.

Results and discussion - Four different inter-helical metal-ion sites located between respectively TM-II and -III, TM-III and -V, TM-III and -VII, and TM-V and VI (Fig. 8, Table in Panel A) were here probed with metal-ion chelator complexes in competition binding experiments against [^{125}I]-substance P in COS-7 cells transiently transfected with the NK1 receptor. An increase in affinity from approx. 10-fold to around 50-fold was observed in the metal-ion site engineered receptors as opposed to the wild-type NK1 receptor for free Zn(II) as well as for Zn(II) in complex with either 1,10-phenanthroline or in complex with 2,2'-bipyridine (Fig.8A). Thus, single to double digit micromolar affinities were obtained for the metal-ion chelator complexes in these metal-ion site engineered receptors, corresponding to affinities observed for lead compounds in general found by conventional chemical screening. In the sites between TM-II and -III and between TM-III and -VII a similar increase in affinity was found for Cu(II) and Cu(II) in complex with the chelators as observed with the zinc-ions. However in the sites between TM-III and -V and between TM-V and -VI no increase or just a marginal increase in affinity was observed for copper and the copper-chelator complexes. Thus, different metal-ions can be exploited in different sites. In Fig.8B is demonstrated that an inter-helical bis-His site, in this case constructed between TM-V and TM-VI, can also be addressed by a metal-ion chelator complex where the ion, in this case Zn(II), is bound in a circular chelator, here cyclam. Cyclam binds Zn(II) with a very high affinity, 3.2×10^{-16} M, which can be noted by the fact that the Zn(II)-cyclam complex has no effect on the wild-type NK1 receptor even at 10^{-3} M conc. i.e. an even smaller effect than the free metal-ion. Thus, the effect of the metal-ion chelator complex on the metal-ion site engineered receptor cannot be caused by the presence of free metal-ions.

The present example demonstrates that metal-ion chelator complexes can bind with suitable affinity, i.e. corresponding to ordinary lead compounds, in different parts of the main ligand-binding crevice of a 7TM receptor. This can be utilized, for example to target the lead compound and thereby subsequently the chemically optimized compound, i.e. the drug candidate, to bind and interact with different parts of the target molecule. In the present case, the metal-ion site between TM-II and -III can be used as anchor point for lead compounds addressing chemical interactions with wild-type residues located in the pocket between TM-I, -II, -III, and VII; whereas the metal-ion sites located between TM-III and -V and TM-V and -VI can be used as anchor points for chelating lead-compounds addressing residues in the pocket between TM-III, -IV, -V, -VI and -VII (see helical wheel diagram in Fig. 8C). The metal-ion site located between TM-III and -VII may in principle be used to address either of these pockets. This approach can be used to deliberately direct the chemical optimization process, i.e. the molecular recognition towards specifically interesting parts of the target protein in order to obtain for example selectivity for a certain receptor subtype or a certain member of a family of related proteins. For example, families of monoamine and adenosine 7TM receptors are generally very highly - if not totally - conserved in the binding pocket for the natural ligand, i.e. the pocket between TM-III, -IV, -V, -VI, and -VII; however, they differ more in the pocket between TM-I, -II, -III, and VII. Conventional drug discovery methods are for various reasons highly biased towards the binding pocket for the natural ligand. The present approach allows for deliberate targeting of the lead compound and thereby also the final drug candidate for allosteric sites, i.e. pockets or epitopes distinct from the one used by the natural ligand.

Example II.2 – Re-engineering of a metal-ion chelator binding site in the 12TM dopamine transporter.

In example I.3 , it was shown that the Zn(II)-bipyridine inhibited dopamine transport in a two-component fashion. This complicated type of interaction could hamper a subsequent further medicinal chemistry optimization of the chelator for high affinity interaction. In this example, the naturally occurring metal-ion site was re-engineered by elimination of one part of the metal-ion binding site and by introduction a new metal-ion binding residue.

Methods - as in example I.3.

Results and discussion - Re-engineering of the metal-ion site in the dopamine transporter was done by eliminating His^{193}, i.e. the residue found in the proposed extra-cellular loop 1, by substitution with a Lys residue and by introduction of an alternative

metal-ion chelating His residue either in exchange for Glu^{396} located at the extra-cellular end of TM-8 or in exchange for Val^{377} located in TM-7 (Norregaard et al. Biochemistry, 26, 15836-46, (2000). Both of these introduced His residues are located in a potentially favorable configuration for participating in metal-ion binding with His^{375} in TM 7. As shown in Fig. 9, in both cases - [H193K;E396H] and [H193K;V377H] - more mono-component interaction curves were obtained for the metal-ion chelator complex in the re-engineered transporter mutants as compared to the wild-type transporter protein. This example demonstrates that a natural metal-ion site can successfully be re-engineered to create a less complex molecular or pharmacological phenotype. In a subsequent medicinal chemical optimization process such re-engineered metal-ion sites will be used in parallel with the natural site during the screening of chemical libraries.

In biological target molecules in general, more than one version of an engineered metal-ion site can in a similar fashion be used in parallel in the screening process in order to exploit the chemical libraries more efficiently. This approach enables each compound to contact, for example the same amino acid side chain located on an opposing transmembrane helix in more than one configuration.

Example II.3 – Probing different metal-ions in an engineered Bis-His TM-V Kappa opioid receptor and evaluating the chelator strength of various metal-ion chelators.

This example illustrates the importance of testing various metal-ions in a metal-ion binding site in for example a 7TM receptor, probing the selectivity of the site. This was done in the Kappa opioid receptor in which a metal-ion binding site was engineered by substituting the endogenous residues for histidine at positions V:01 and V:05 in TM-V (i.e. [D223H;K227H]).

Methods - Mutations were created in the rat Kappa opioid receptor cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59). The Kappa opioid receptor cDNA was expressed in COS-7 cells. Two days after transfection whole cells were assayed with respect to binding of radioactively labeled [^{3}H]-Diprenorphine in displacement with Diprenophine, or various metal-ions. The assay was typically performed in 12 or 24 well plates. On the day of assay, the cells were washed with binding buffer (50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 % BSA, 0.1 % and Bacitracin (100 mg/ml). Unlabelled competitor ligand and radioligand (ca. 50 pM) was added to the cells in binding buffer and incubation continued for 3 hours at 4 °C. The assay was terminated by washing of the cells and lysis. The assay was performed in duplicate.

For evaluation of the relative chelator strength for Zn(II), a fluorescence based assay was performed. Briefly, the chelator of choice was dissolved in 20 mM sodiumphosphate buffer pH 7.4, 100 mM NaCl, 200 micromolar mercaptoethanol, 20 nM $ZnCl_2$ and 1 micromolar FluoZin-3 (Molecular Probes) and incubated for two hours at room temeperature after which the fluorescence of fluozin-3 was measured in a NovoStar reader (BMG).

Results and discussion – As shown in Fig. 19, the engineered metal-ion site, shows a clear selectivity for certain metal-ions over others. For example, whereas Zn(II) has an IC50 of 1.1 micromolar and Cu(II) an IC50 value of 4 micromolar, Gd(II) is a significantly lower apparent affinity of 42 micromolar.

The selectivity of a metal-ion site in a biological target may be used in combination with the selectivity displayed by various metal-ion chelators, Fig. 10, to fine tune the binding of the complex to the metal.ion site in the biological target at for example the desired affinity range.

The experiments presented in this section demonstrate that metal-ion chelator complexes can act as blockers of the function of biological target molecules - in these cases of either 7TM receptors or 12TM transporter proteins - through binding to metal-ion sites introduced by mutagenesis. Furthermore, these compounds can bind with similar affinity as lead compounds found by conventional drug screening techniques. Thus, these metal-ion chelators can function as lead compounds in a chemical optimization process to obtain high affinity compounds acting as drug candidates.

III. Increasing The Affinity / Potency / Efficacy Of The Metal-Ion Chelator Complexes Through Chemical Modifications Of The Chelator Molecule

In the present collection of examples, the metal-ion chelators are considered as being bi-functional compounds, i.e., being composed of a metal-ion chelating moiety and a variable chemical moiety which interacts positively or negatively – depending on the chemical recognition - with spatially surrounding parts of the biological target molecule to which the chelator binds through either a natural or an engineered metal-ion site.

Example III.1 – Structure-activity relationship of antagonist metal-ion complexes in the leukotriene LTB4 7TM receptor.

As discussed in examples I.1 the human leukotriene LTB4 receptor has a metal-ion site located between Cys^{93} and Cys^{97}, located in TM-III.

Methods – as in examples I.1.

Results and discussion - A small library of commercially available 1,10-phenanthroline analogs in complex with Cu(II) were tested in competition binding against [^{3}H]-LTB4 on the LTB4 receptor expressed in COS-7 cells. Interestingly, a significant increase in affinity was observed for compound TM-270, whereas a significant decrease in apparent affinity was observed for compound TM-401, demonstrating SAR on the LTB-4 receptor (Fig. 11).

Example III.2 – Structure-activity relationship of antagonistic metal-ion complexes in the metal-ion site engineered tachykinin NK1 7TM receptor

The tachykinin NK1 receptor, which currently in the industry is a major putative target for the development of anxiolytic, antidepressive, as well as anti-emetic drugs, is here used as an example of a biological target molecule, in which an engineered metal-ion site can be used as an anchor point for the discovery and development of antagonistic drug candidates. As demonstrated in example II.1 a number of metal-ion sites could be built into the NK1 receptor and addressed by metal-ion chelator complexes competing for binding against radioactive substance P through interactions at different sites in the main ligand-binding pocket of the receptor, depending on the location of the metal-ion. Here, structure-activity relationships are demonstrated for a series of chelator analogs in two of these sites, i.e. the site between V:05 and VI:24 and the site between III:08 and VII:06.

Methods – as in example II.1.

Results and discussion – Many of the chemical variations of the variable part of the chelator were tolerated in the structure of the NK1 receptor when bound to the engineered metal-ion sites in complex with Zn(II). However, as demonstrated in Fig 12, clear differences were observed for some of the analogs in the two selected sites. Thus, 2,9-bis(trichloromethyl)-1,10-phenanthroline (compound 135) and 1,10-phenanthroline-5,6-dione (compound 175) bound 6- and 10-fold better than 1,10-phenanthroline in the [HisV:05,HisVI:24] site, but almost similar to 1,10'-phenanthroline in the [HisIII:08;CysVII:06] site - all in complex with Zn(II). In contrast the 5-phenyl-1,10-phenanthroline (compound 134) was 7-fold more potent in the [HisIII:08;CysVII:06] site than phenanthroline but only slightly more potent in the [HisV:05,HisVI:24] site - again all in complex with Zn(II). It should be noted here, that 5-phenyl-1,10-phenanthroline

(compound 134) bound like 1,10'-phenanthroline in the galanin receptor, but was totally inactive in the leukotrien LTB4 receptor (see Fig. 11).

This example together with the previous example demonstrate, that relatively minor chemical modification of the variable, "non-metal binding" part of the chelator molecule can alter the recognition and antagonistic property of the metal-ion chelator complex both in biological target molecules having naturally occurring metal-ion sites as well as in molecules into which metal-ion sites have deliberately been engineered. Importantly, increases in affinities are observed demonstrating that the metal-ion chelators can be utilized as lead compounds in a drug discovery process towards high affinity compounds.

Example III.3 – Structure-activity relationship and dependency of specific metal-ion chelating residues of *agonist* metal-ion complexes in the metal-ion site engineered beta$_2$-adrenergic 7TM receptor.

It is generally known in the field that while it is possible to find *antagonistic* lead compounds and optimize these for high affinity through medicinal chemistry efforts in many biological target molecules, it is generally much more difficult to find and develop *agonist* compounds, that is compounds, drug candidates, which activate the biological target molecule. The present example demonstrates how an engineered agonistic metal-ion site can be used as anchor-point for the development of *agonists* in a 7TM receptor. Furthermore the present example demonstrates the importance of testing various combinations of chelating aminoacids in the same positions when engineering a metal-ion bindings site, as the specific positions, of which the site is composed, may only define a metal-ion binding site for some combinations (e.g. His-Cys or Asp-Cys) and not others (e.g. His-His, Glu-His etc.).

Methods – Mutations were created in the beta2-AR cDNA by the PCR-directed overlap-extension method (Ho et al. (1989) Gene 77: 51-59). The beta2-AR cDNA was expressed by transient transfection into COS-7 cells. Two days after transfection the cells were assayed for intracellular levels of basal and ligand-induced cyclic AMP. The assay employed is essentially as described in Solomon et al (Anal.Biochem. (1974) 58: 541). Labelled adenine ([^{3}H]adenine, Amersham TRK311) was added to cells seeded in 6-well culture dishes. The following day the cells were washed twice with HBS buffer [25 mM Hepes, 0.75 mM NaH_2PO_4, 140 mM NaCl (pH 7.2)] and incubated in buffer supplemented with 1 mM 3-isobutyl-1-methylxanthine (Sigma I-5879). Agonists were added and the cells were incubated for 30 min at 37 °C. The assay was terminated by placing the cells on ice

and aspiration of the buffer followed by addition of ice-cold 5% trichloroacetic acid containing 0.1 mM unlabelled camp (Sigma A-9062) and ATP (Sigma A-9501). Cyclic AMP was then isolated by application of the supernatant to a 50W-X4 resin (BioRad) and subsequently an alumina resin (A-9003; Sigma) eluting the cyclic AMP with 0.1 M imidazole (Sigma I-0125). Determinations were done in duplicate. For the wash experiment the [F289C;N312C]β_2-AR construct was stimulated with 100 μM copper-(2,2'-bipyridine)3 complex at time zero; after 10 minutes cells were washed two times whereafter half of the cells were either re-challenged with 100 μM copper-(2,2'-bipyridine)3 or with buffer. The basal cAMP accumulation was monitored in parallel. The level of cAMP production was sampled for each 5 minute interval.

Results and discussion – The inventors have previously demonstrated that Cys-substitution of Asn^{312} (AsnVII:06) in TMVII in the beta2-adrenergic receptor creates a bi-dentate metal-ion binding site with AspIII:08 at which metal-ion chelator complexes such as 1,10-phenanthroline and 2,2'-bipyridine in complex with either Zn(II) or Cu(II) can bind and act as agonists for the receptor (Elling et al. PNAS 1999, 15: 6213-6219). As shown in Fig. 13 and Fig. 20 an extended version of this site including also a substituted residue, Phe^{289} (PheVI:16) located in the important TM-VI, or a site only bridging positions III:08 in TM-III and position VI:16 in TM-VI, metal-ion chelator complexes, in this case Cu(II)-1,10-phenanthroline and Cu(II)-2,2'-bipyridine display higher agonistic efficacy and in some cases potency, than in the TM-III to TM-VII site. The free metal-ion or the chelator by itself has no stimulatory effect in the metal-ion-site engineered receptor (Fig. 20). That the agonistic effect of the metal-ion chelator complex is not caused by some kind of covalent modification of the receptor - for example oxidation – is shown in Fig. 14A, where a simple washing experiment demonstrates how the stimulatory effect quickly disappears, when the metal-ion chelator is removed, while the stimulation continues if the metal-ion chelator complex is re-added. When a library of bipyridine analogs, substituted either in the 3, 4 or 5 position were tested for agonistic activity in the [F289C] beta-2 adrenergic receptor, many were found not to be active, or to be less active than 2,2'-bipyridine, Fig. 15C, while some were shown to be as potent as bipyridine itself (Fig . 14B and 15C). Importantly, a compound such as 2,2'-di(4-(benzimidazol-2-yl)-quinoline),(compound 85) was found to stimulate signal transduction as determined in cAMP accumulation in the metal-ion site engineered receptor with a 21-fold improved potency, i.e. EC_{50} = 470 nM as compared to 2,2'-bipyridine. Furthermore, a number compounds such as for example copper-(TM-325)2 had a higher efficacy compared to copper-(2,2'-bipyridine)2, Fig.15C.

The importance of testing various combinations of chelating amino acids in the same positions, when engineering a metal-ion binding site is shown in Fig. 20 and Fig. 13A. Whereas a given combination of aminoacids in positions 113 (III:08) and 312 (VII:06), for example histine, cysteine respectively define an agonistic metal-ion binding site, other combinations, for example histidine, histidine respectively, does not. Similarly, whereas engineering of an agonistic metal-ion binding site composed of chelating residues at positions 113 (III:08) and 289 (VI:16) is successful with the combination aspartate (utilizing the naturally occurring aspartate 113 (III:08)) and cysteine respectively, the combination histidine, histidine respectively is unsuccessful. Furthermore, whereas more than one combination of amino acids may successfully define a metal-ion binding site at the same positions, the individual successful combinations may have different properties reflected in for example the specific pharmacology. In Fig. 20 this is demonstrated by the differing agonistic efficacy and cation selectivity of constructs involving postion 113 (III:08) and position 289 (VI:16).

This example demonstrates, that the variable, non-metal-ion binding part of the chelators can be modified to create nanomolar affinity agonists in metal-ion site engineered biological target molecules. Such a compound could serve as an intermediate "chemical stepping-stone" in the process of developing high affinity agonists for the metal-ion site engineered receptor. And, similarly agonistic metal-ion sites can be engineered into other 7TM receptors and other biological target molecules in general to serve as anchor points for the initial identification as well as the initial optimization process for agonist leads for such target molecules. Furthermore, this example demonstrates the importance of testing various combinations of chelating amino acids in the same positions when engineering a metal-ion binding site, as some combinations may successfully define a site and other combinations may not, and as the various successful combinations may have different properties, reflected in for example their specific pharmacology or cation selectivity.

Example III.4 – Structure-activity relationship of antagonistic metal-ion complexes in a soluble protein, the enzyme factorVIIa (FVIIa)

The previously presented examples have all represented membrane proteins, which obviously constitute a very large group of biological target molecules for medical drugs. In the present example, Factor VIIa, i.e. the active form of the FVII protease involved in the coagulation cascade is used to demonstrate that metal-ion chelator complexes can modulate the function of a soluble protein, in this case an enzyme which is known to

possess an appropriate, allosteric metal-ion site (Dennis et al. Nature (2000) 404: 465-470).

Method – The amidolytic activity of Factor VIIa (FVIIa) was measured by the incubation of 2.5 µl FVIIa (100 nM final concentration, obtained from American Diagnostica), 2.5 µl ligand and 4 µl substrate (10 mM, S2288 obtained from Chromogenix) in 42.5 µl buffer (50 mM Hepes pH 7.4, 1 mM $CaCl_2$, 100 mM NaCl, 0,02% Tween 20). The assay was performed in 96-well plates (Costar). Incubation was performed at room temperature for five hours with absorbance read every 10 minutes.

Results and discussion – As shown in Fig. 16A, 2,2'-bipyridine without metal-ions has no effect on the activity of FVIIa; however in complex with Zn(II), 2,2'-bipyridine inhibits the enzymatic activity with a 100 micromolar affinity. Many bipyridine analogs act with a similar potency as the basic chelator, however for example Zn(II)-4,4'-di-terbutyl-2,2'-dipyridyl (compound 180) inhibits FVIIa enzyme activity with an 8.5-fold *increased* potency as compared to Zn(II)-bipyridine (Fig. 16A). In contrast Zn(II)- 4,4'-di-terbutyl-2,2'-dipyridyl (compound 180) inhibits LTB4 binding to the LTB4 receptor with a potency which is 10-fold *lower* than Zn(II)-bipyridine alone (Fig. 16B). As shown in Fig. 16C, 1,10-phenanthroline had no effect on FVIIa activity by itself, however in complex with Zn(II) 1,10-phenanthroline inhibits the enzyme activity with a potency of 110 micromolar. As with 2,2'-bipyridine, many phenanthroline analogs act with a potency similar to or lower than 1,10-phenanthroline itself (data not shown); however, for example 2,9-bis(trichloromethyl)-1,10-phenanthroline in complex with Zn(II) inhibits FVIIa activity with increased potency as compared to Zn(II)-1,10-phenanthroline (Fig. 16C).

Most enzyme inhibitors act by binding at - or near by - the active site of the target molecule. However, as recently demonstrated for FVIIa, very efficient inhibition can be obtained also by binding instead at exosites or allosteric sites located far away from the active site in the biological target molecule (Dennis et al. Nature (2000) 404: 465-470). The method described here can be utilized to specifically target the lead compound and thereby the final drug candidate to act at allosteric sites in the target molecule, as the binding site is determined by the site at which the anchoring metal-ion site is engineered. Inhibition of enzymes and proteins in general at allosteric sites is particularly interesting since the active site often is relatively similar in enzymes belonging to a particular protein family, for example kinases or phosphatases, which means that it can be difficult to obtain selectivity of drugs acting at the active site. This is not the case with drugs acting at allosteric sites.

Example III.5 – Structure-based optimization of metal-ion chelators for secondary interactions in the CXCR4 receptor and other biological target molecules.

The previous examples in this session have demonstrated, that it is possible to obtain both decreased, but importantly, also *increased* affinity by modifying the variable, non-metal binding part of metal-ion chelators, which in various biological target molecules bind to either natural or engineered metal-ion sites. These examples were gathered mainly from screenings of commercially available, small libraries of chelator analogs. In the present example it is described how the process of increasing the affinity or potency of the metal-ion chelator can be performed in a deliberate structure based fashion in this case through the establishment of a charge-charge interaction. The metal-ion-mediated binding of the metal-ion chelator is here considered as being the "primary interaction point" or the anchor point, while the subsequent establishment of other chemical interactions is considered to be "secondary interaction points".

Methods –The cDNA coding for, for example the CXCR4 chemokine receptor can be expressed in COS-7 cells as described for other 7TM and 12TM proteins previously. Metal-ion sites may be engineered through PCR-directed mutagenesis and the functional activity of the receptor be tested for instance by (established) binding experiments employing the radiolabelled ligand, [^{125}I]-SDF1α.

Results and discussion – The inventors have demonstrated that Asp^{171} (AspIV:20) located at the extracellular end of TM-IV on the face pointing inwards, towards the main ligand binding crevice of the CXCR4 receptor is exposed and can be used as attachment site for the positively charged cyclam ring of non-peptide bicyclam antagonists for this receptor. Metal-ion binding sites will be introduced in the CXCR4 receptor in the spatial vicinity of AspIV:20 by introduction of a His residue at position V:01 which will form a bis-His metal-ion binding site with the naturally occurring HisIII:05 in the CXCR4 receptor - as previously demonstrated in the NK1 receptor (Elling et al. EMBO J. (1996) 15: 6213-6219). Similarly an intra-helical bis-His site will be introduced between residues V:01 and V:05 through introduction of two His residues at these positions and between III:05 and IV:24 through His substitution at position IV:24. Thus three metal-ion sites will be constructed all within few Å's of AspIV:20 (see helical wheel diagram in Fig. 17). A small library of 1,10-phenanthroline, 2,2'-bipyrdine and 8-mercaptoquinoline analogs will be obtained or synthesized in which amino-methyl, amino-ethyl, amino-propyl, and aminobutyl will be placed in either the 2, 3, 4, or 5 positions and a similar small library

where the same substituents will be placed in either the 3, 4, or 5 position of bipyridine will similarly be constructed. In a typical experiment, these libraries of amino-substituted chelators will be tested in complex with either Zn(II) or Cu(II) in the metal-ion-site engineered CXCR4 receptors, and the compounds ability to inhibit the binding of ^{125}I-SDF1a or the binding of [^{125}I]-12G5 monoclonal antibody or the ability of the compounds to inhibit the signal transduction mechanism induced by SDF-1a will be tested as performed for metal-ion chelators in the previous examples described above. Due to the spatial proximity as well as the relative conformational flexibility of the system, several of these compounds will in several of the sites have the opportunity of forming a salt-bridge between the amino function of the amino-substituted metal-ion chelator and the carboxylic acid function of Asp^{171} (AspIV:20). This formation of a secondary interaction will be quantified as an increased affinity or an increased potency of the metal-ion complex of the amino-substituted chelator in comparison to the corresponding metal-ion complex of the non-substituted phenanthroline or dipyridine. Due to the relatively high energy in the charge-charge-interaction a considerable increase in affinity or potency will be observed. The molecular interaction mode of the amino-substituted chelator(s) will be confirmed through mutational substitutions of Asp^{171} with Asn, Ala and other residues. Depending on the structure of the most optimal amino-substituted analog(s) a second and third round of analogs will be synthesized which conceiveably will present an appropriate basic moiety in a more conformationally constrained fashion.

These mini-libraries of amino-substituted metal-ion chelators can be utilized in several biological target molecules, which present Asp or Glu residues in an appropriate fashion. For example, in the CXCR4 receptor Asp^{262} (AspVI:23) is equally available as Asp^{171} for interaction as previously described (Gerlach et al.). Similarly AspIII:08 is conserved among monoamine receptors and, for example opioid and somatostatin receptors and this residue is a known interaction point for amine functions (Strader et al (1991) 266: 5-8). These and other acidic, potential secondary interaction points for amino-substituted metal-ion chelators can be addressed through construction of a small number of metal-ion sites placed in their spatial vicinity - as described above for Asp^{171} (AspIV:20). Similarly amino-functions in a biological target molecule – for example, epsilon amino groups of Lys residues –can be addressed by, for example mini-libraries of tetrazol substituted metal-ion chelators. As described, charge-charge interactions will initially be pursued for establishing secondary interactions for the metal-ion chelator lead compounds. However, other types of weaker interactions such as hydrogen-bonds, amino-aromatic interactions, aromatic-aromatic interactions, aliphatic hydrophobic interactions, cation-pi interactions,

van der Walls interactions etc. will also be exploited in a similar, systematic fashion as described above for the charge-charge interactions.

Example III.6 – Increasing the affinity of the metal-ion chelator in the [HisV:01;HisV:05] engineered LTB4 receptor

This example demonstrates how complexes with increased affinities may be identified on an engineered metal-ion binding site in the leukotriene B4 receptor.

Methods – see example I.1

Results and discussion – A library of approximately 500 2,2'-bipyridine analogues were synthesized and screened in combination with copper on the LTB4 receptor containing an engineered metal-ion binding site at the top of TM-V, [HisV:01;HisV:05]. From this screen, a compound was identified with a 50-fold increase in affinity compared to the unsubstituted scaffold, 2,2'-bipyridine, Fig. 18. Importantly, the compound had no increase in affinity on the wildtype LTB4 receptor, Fig 18. This illustrates how the affinity of a metal-ion complex may be increased by chemical modification of the chelator molecule, enabling secondary interactions to be identified.

In the present section, a 7TM receptor is for convenience used as an example of a biological target molecule. In this system, very useful molecular models are available, which have been refined and have allowed for, for example the construction of intra- and especially inter-helical metal-ion sites. However, due to lack of, for example an array of suitable X-ray structures of this or similar targets in complex with agonists and antagonists it is not possible to apply classical structure-based drug design methodology in full. Nevertheless, for example in these membrane proteins the present method does to a certain degree compensate for the lack of knowledge of the detailed 3D structure of the target molecule by anchoring the lead compound and thereby creating a fix-point for the subsequent medicinal chemical optimization point guided by the molecular models.

The approach described above could be further helped and guided by detailed knowledge of the 3D structure(s) of the biological target molecule, preferentially determined in complex initially with the un-substituted metal-ion chelator and subsequently in complex with the chemically modified metal-ion chelator in which attempts have been made to establish first one secondary interaction and subsequently further secondary or tertiary interactions. For some biological target molecules such as soluble proteins this can be

achieved through for example crystallization and standard X-ray analysis procedures or through, for example NMR analysis of the complex in solution again using standard procedures. Here, the method can take advantage of methods developed for structure-based drug discovery in general. This would make it possible to apply classical structure-based approaches such as structure-based library design for the establishment of secondary and tertiary interaction sites for the lead compound in the target molecule. However, it should be noted, that a major advantage and difference of the present method is, that the lead compound is anchored to a particular site and thereby to a certain degree in a particular conformation in the biological target molecule through binding to the bridging metal-ion site while the compound is being optimized for chemical recognition with the target molecule.

Also it should be noted that through the application of a more-or-less flexible spacer in between the metal-ion chelating moiety and the so-called variable chemical moiety of the test compound it becomes possible to probe for interaction or binding to structurally and functionally interesting epitopes of the biological target molecule with variable chemical moieties, which due to their intrinsic low affinity would not be detectable in the analytical systems on their own; but, which - due to the local high concentration of these created by the binding of the tethering metal-ion chelating moiety to the metal-ion site - now are detected.

Example III.7 - Improving the affinity of a stable test compound for a metal-ion site engineered receptor previously employed for creating a genetically modified test animal through establishment of second site interactions in the receptor by chemically modifications of the chelator.

In the previous example it was demonstrated that stable test compounds – i.e. metal-ion chelator complexes - acting as efficient inhibitors in metal-ion site engineered receptors can be made through the use of, for example Pd(II) as the metal-ion. In order for a test compound to efficiently and selectively block the function of a metal-ion site engineered biological target molecule in an in vivo setting it needs besides being stable also to have a sufficient high affinity for the metal-ion site engineered receptor. In the present example it is demonstrated that the affinity of such a stable metal-ion chelator complex can be increased considerably through chemical modification of the side-chain of the chelator establishing second site interactions with the receptor.

Method – see example I

Results – As shown in Fig. 21 (upper panel to the left), bipyridine or rather 4,4-dimethyl-bipyridine in complex with Pd(II) does not affect the signaling of the wildtype RASSL receptor in response to the non-peptide agonist ICI199,441 and it has an inhibitory potency above 10^{-6} M in the RASSL receptor with an engineered metal-ion site between the natural AspIII:08 and the mutated CysVII:06 (the results for the Pd(II) complex with 4,4-dimethyl-bipyridine is shown in the figure to indicate that minor substitutions to the bipyridine chelator had no beneficial effect on the potency of the metal-ion chelator complex). When the bipyridine chelating moiety is decorated with a ethoxycarbonylcyclohexyl moiety connected via an amide bond (compound 433) then its Pd(II) complex is still inactive in the wildtype RASSL receptor, however now the potency of the Pd(II)complex of compound 433 has a potency of 200 nM (Fig. 21, lower left panel). Thus, the side chain which has been placed on the bipyridine cchelator has established second site interactions with the metal-ion site engineered RASSL receptor which has increased its affinity as here determined by the more than 100 fold increase in its potency in blocking the ICI199,441 stimulation of the receptor. This example demonstrates the general principle in increasing the affinity of a test compound through the establishment of second site interactions by chemical modifications of the metal-ion chelator. This can be achieved through non-covalent interactions as shown in the present example or through the establishment of covalent bonds, for example with a natural or an introduced, reactive Cys residue.

Example III.8 – Structure-based optimization of metal-ion chelators to use as antagonists in "pharmacological knock-out" experiments.

The approach described in the previous examples will be used as (a) step(s) in the drug development process in general to increase the affinity of lead compounds for the biological target molecule through establishment of chemical recognition between the ligand and structural elements found in the wild-type target molecule, i.e. in the unmodified vicinity of the engineered metal-ion site. However, the method will also be used for example to increase the affinity and specificity of metal-ion chelator compounds to be used in pharmacological knock-out applications. This procedure has in principle been described previously (Elling et al. (1999) Proc.Natl.Acad.Sci.USA 96: 12322-12327); however only for basic metal-ion chelating agents. Briefly, the method is based on the introduction of a silent metal-ion site in a potential drug target, i.e. creation of a metal-ion site in which the mutations do not affect the binding and action of the endogenous ligand for the receptor. When such a metal-ion site engineered receptor is introduced into an

animal by classical gene-replacement technology, i.e. exchange of the endogenous receptor with the metal-ion site engineered receptor, then the animals will develop normally without any development of compensatory mechanisms, which otherwise frequently impair the interpretations of the phenotypes in classical gene knock-out technology. In the adult animals or whenever it is found appropriate the animals are then treated with an appropriate metal-ion-chelating agent which then will act as an antagonist and turn off the function of the metal-ion site engineered receptor. Currently, this approach is impaired by the fact, that the generally available metal-ion chelating agents only will bind with at best micromolar affinity to the metal-ion site engineered biological target molecule, which will give similar micromolar or lower antagonistic potencies. These relatively low potencies and the relative low specificity of the basic test compounds impairs the general applicability of the technology due to simple pharmacokinetic and toxicology problems.

By applying the technology described in the previous example and in the previous examples in general, it will be possible to increase the affinity of metal-ion chelators significantly, which will make it considerably more easy to reach therapeutic, efficient antagonistic concentrations of the metal-ion chelator in the animals and also to increase the "therapeutic window" due to the higher degree of selectivity of the compounds caused by the establishment of more than one molecular interaction point. Establishment of just a single suitable charge-charge interaction will increase the affinity of the metal-ion chelator by 10 to 100-fold or more. This will be performed as an example in the so-called RASSL a modified kappa-opioid receptor, which previously has been used in gene-knock out experiments (Redfern et al. Nat. Biotechnol. (1999) 17:165-169). By introduction of metal-ion sites, for example between TM-V and TM-VI or between TM-VI and TM-VII or between TM-II and TM-III or between TM-III and TM-VII in a kappa-opiod RASSL molecule and through screening of, for example the mini-library of amino-substituted metal-ion chelators it will be possible to select a nano-molar affinity antagonist because of the formation of a secondary charge-charge interaction with AspIII:08, i.e. the Asp in TM-III corresponding to the amine-binding Asp in monoamine receptors.

IV. Optimization Of Compounds On The Wild-Type Biological Target Molecule

In the case, where the initial binding of the metal-ion chelator was obtained through mutational introduction of an anchoring metal-ion site in the biological target molecule, a final step of optimization will have to be performed to obtain high affinity binding or potency on the wild-type target molecule without the metal-ion bridge. Through the

methods described in the previous experiments, the metal-ion chelator lead compound will gradually be optimized for interactions with chemical groups in the biological target molecule spatially surrounding the metal-ion site - i.e. interactions with chemical groups found also in the wild-type target molecule. Thus, the test compound will gradually increase its affinity not only for the metal-ion site engineered molecule but also for the wild-type biological target molecule. When two to three secondary interaction points have been established, the affinity of the test compound for the wild-type target molecule, which is being tested in parallel with the metal-ion site engineered molecule, will have reached micro-molar affinities, i.e. a lead compound on the wild-type target molecule has been created. At this point one or more of the following three approaches will be followed: 1) structure-based further chemical optimization of the compound in general aiming at improving recognition at various known chemical moieties of the target molecule; 2) structure-based further chemical optimization of the compound at which the "metal-ion site bridge" is exchanged by a more classical type of chemical interaction with the residue(s) which had been modified to create the metal-ion site in the biological target molecule. Here advantage can be taken of the fact that the geometry of the metal-ion site anchor is well known in general and, that relatively limited structure-based libraries can be established to create a new type of interaction; 3) further chemical optimization of the compound through more-or-less random generation of chemical diversity in general in the compound.

The above-given examples describe specific methods that can be employed to practice the present invention. Based on the details given a person skilled in the art will be able to devise alternative methods at arriving in the same information using the concept of the invention. However, the examples are not to be construed to limit the invention in any way.

The following examples illustrate the use of metal-ion chelators in a target validation process.

Structure-based optimisation of metal-ion chelators to use as antagonists in "pharmacological knock-out" experiments

The main method of the present invention is – briefly - based on the introduction of a silent metal-ion site in a potential drug target, i.e. creation of a metal-ion site in which the mutations do not affect the binding and action of the endogenous ligand for the receptor and introduction of this into a thereby genetically modified test animal. The generation of a

silent antagonistic metal-ion switch has previously been performed, for example in the kappa opioid receptor (Thirstrup et al., (1996) J. Biol. Chem. 271, 7875-7878). The kappa opioid receptor has also been used as the basis for creating so-called RASSL's i.e. a receptor which is only activated by synthetic compounds and not by endogenous hormones and transmitter and therefore when introduced into a genetically modified test animal it can selectively be activated by the "drug" (Redfern et. al. (1999) Nature Biotechnology 17:165-169). It should be noted that the technology described in that paper does not involve creating a binding site for the employed "drug" and the use of metal-ion sites in the modification of the receptor. However, the paper demonstrates that the technology of creating a genetically modified test animal in which an endogenous biological target molecule is exchanged with a protein-engineered version of this biological target molecule and in which the target molecule is specifically turned on or off by drug-like substances leading to relevant physiological, pharmacological changes in the animal, has already been enabled. In that context that part of the technology is used merely to study the effect of turning a particular signal transduction pathway on in a particular tissue at a particular time point. The present invention aims at probing for the physiological function and pharmacological potential of particular biological target molecules as such – target validation.

EXAMPLE 50

Improving the stability and affinity of a test compound for a metal-ion site engineered into a receptor previously employed for creating a genetically modified test animal

In order for a test compound, a metal-ion chelator complex to efficiently and selectively block the function of a metal-ion site engineered biological target molecule it needs to have a sufficient high affinity for the metal-ion site engineered receptor. Also, in order to ensure that the test compound reaches the metal ion engineered biological target molecule as an intact complex in the genetically modified animal, it is important that the dissociation of the metal ion chelator complex is slow. In the present example a high affinity inert metal-ion chelator complex is obtained by using palladium, Pd(II) as the metal ion in complex with a chemically improved analog of phenanthroline.

Method – The cDNA coding for the RASSL receptor or for a RASSL receptor in which a metal-ion site had been introduced by introducing a Cys residue in position VI:06 as

previously done in the beta2-adrenergic receptor (C.E. Elling et al, *PNAS 96*, 1999, pp. 12322-12327), the bidentate metal-ion site is located between the natural AspIII:08 and the introduced CysVII:06) was introduced into COS-7 cells by the standard calcium phosphate transfection method. One day after transfection COS-7 cells were incubated for 24 hours with 5 μl Ci of [^{3}H]-*myo*-inositol (Amersham, PT6-271) in 1 ml medium supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin per well. Cells were washed twice in buffer, 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 0.05 % (w/v) bovine serum; and were incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37°C for 30 min. After stimulation with various concentrations of peptide for 45 min at 37°C, cells were extracted with 10 % ice-cold perchloric acid followed by incubation on ice for 30 min. The resulting supernatants were neutralized with KOH in HEPES buffer, and the generated [^{3}H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resin. Determinations were made in duplicates.

Results – As shown in Fig. 21, phenantholine in complex with Zn(II) inhibits the signaling of the metal-ion site engineered kappa opioid receptor RASSL with a potency of 99 μM. Introduction of just a single chloro function in the 5 position of phenanthroline increases the affinity by approximately 10 fold (12 μM) – indicating that just minor suitable modifications of the test compound can significantly increase the usefulness of such compounds as antagonists of metal-ion site engineered receptors. Similarly, introduction of just a single amino function in the 5 position increases the affinity by 15 fold (6.4 μM) (Fig. 22). However, these modifications does not improve the pharmakokinetic properties in respect of allowing the intact chelate to reach the receptor. As described previously Zn(II) has the disadvantage of being a labile metal-ion with a T½ of dissociation from phenanthroline analogs of approximately one tenth of a second. In order to increase the T½ for the metal- ion chelator complex (the chelate) we used metal ions e.g. Ru(II), Pd(II), Rh(II), that are characterized by forming inert complexes, i.e. complexes with slow dissociation rates, with bipyridine and phenanthroline.

As shown in Fig. 23, the 5-chloro-analog of phenanthroline in complex with Pd(II) inhibits the agonist induced signaling, i.e. IP3 accumulation, of the metal-ion site engineered RASSL receptor with a potency of 2 μM, i.e. five-fold higher than the same compound in complex with Zn(II). Importantly, the Pd(II) compound was not able to inhibit the agonist induced signaling of the unmodified RASSL receptor even at concentrations up to 100 μM, i.e. the receptor without an engineered metal-ion site, indicating that the compound act by

binding to the engineered metal-ion site and that a relatively high degree of specificity of the compound has been achieved (Fig. 24).

The Pd(II) not only dissociate very slowly, i.e. in fact it binds covalently to the small molecule chelator compound, it also dissociates slowly from the metal ion site in the engineered receptor, i.e. it acts as an irreversible antagonist, which will be very useful and efficient as an antagonist also in the genetically modified animal. In Fig.25 are shown how the small molecule, non-peptide agonist ICI199.441 dose-dependently stimulates IP3 accumulation in the metal ion site engineered RASSL receptor (square symbols). However, if the cells are incubated 30 min with the Pd(II)-compound and afterwards are washed repeatedly, before the receptors are stimulated with ICI199.441 then both the potency and the efficacy of the agonist is severely impaired. It is not possible to wash away the inhibitory effect of Pd(II)5-chloro-phenanthroline which indicates that the metal-ion chelator complex binds irreversibly to the metal-ion site in the receptor.

The high potency of the simple Pd(II) compound indicates, that other chelators, optimized for secondary interaction with the receptor, in complex with a metal-ion such as Pd(II) will be useful for in vivo testing in genetically modified test animals and, that high potent antagonists for these modified receptors can be achieved.

EXAMPLE 51

Improving the affinity of a test compound for a metal-ion site engineered into a receptor

The classical gene knock-out for the CXCR4 chemokine receptor is embryological lethal, meaning that it is impossible to perform target validation on this receptor through ordinary knock out technology.

Methods –The cDNA coding for, for the CXCR4 receptor can be expressed in COS-7 cells as described for other 7TM and 12TM proteins previously. Metal-ion sites may be engineered through PCR-directed mutagenesis and the functional activity of the receptor be tested for instance by (established) binding experiments employing the radioactive ligand, [^{125}I]-SDF1α.

Results and discussion – The inventors have demonstrated that Asp^{171} (AspIV:20) located at the extracellular end of TM-IV on the face pointing inwards, towards the main ligand

binding crevice of the CXCR4 receptor is exposed and can be used as attachment site for the positively charged cyclam ring of non-peptide bicyclam antagonists for this receptor. Metal-ion binding sites will be introduced in the CXCR4 receptor in the spatial vicinity of AspIV:20 by introduction of a His residue at position V:01 which will form a bis-His metal-ion binding site with the naturally occurring HisIII:05 in the CXCR4 receptor - as previously demonstrated in the NK1 receptor (Elling et al. EMBO J. (1996) 15: 6213-6219). Similarly an intra-helical bis-His site will be introduced between residues V:01 and V:05 through introduction of two His residues at these positions and between III:05 and IV:24 through His substitution at position IV:24. Thus three metal-ion sites will be constructed all within few Å's of AspIV:20 (see helical wheel diagram in Fig. 26). Bipyridine analogs will be synthesized in which amino-methyl, amino-ethyl, amino-propyl, and aminobutyl will be placed in either the 3, 4, or 5 position of bipyridine. In a typical experiment, amino-substituted chelators will be tested in complex with either Zn(II) or Cu(II) or Ru(II) in the metal-ion-site engineered CXCR4 receptors, and the compounds ability to inhibit the binding of ^{125}I-SDF1 or the binding of [^{125}I]-12G5 monoclonal antibody or the ability of the compounds to inhibit the signal transduction mechanism induced by SDF-1a will be. Due to the spatial proximity as well as the relative conformational flexibility of the system, several of these compounds will in several of the sites have the opportunity of forming a charge-charge interaction between the amino function of the amino-substituted metal-ion chelator and the carboxylic acid function of Asp^{171} (AspIV:20). This formation of a secondary interaction will be quantified as an increased affinity or an increased potency of the metal-ion complex of the amino-substituted chelator in comparison to the corresponding metal-ion complex of the non-substituted phenanthroline or dipyridine. Due to the relatively high energy in the charge-charge-interaction a considerable increase in affinity or potency will be observed. The molecular interaction mode of the amino-substituted chelator(s) will be confirmed through mutational substitutions of Asp^{171} with Asn, Ala and other residues. Depending on the structure of the most optimal amino-substituted analog(s) a second and third round of analogs conceivably with conformationally constrained arms to the amino function and will present an appropriate basic moiety in a more conformationally constrained fashion.

These amino-substituted metal-ion chelators can be utilized in several biological target molecules, which present Asp or Glu residues in an appropriate fashion. For example, in the CXCR4 receptor Asp^{262} (AspVI:23) is equally available as Asp^{171} for interaction as previously described (Gerlach et al.(2001) J.Biol.Chem in press). Similarly AspIII:08 is conserved among monoamine receptors and, for example opioid and somatostatin receptors and this residue is a known interaction point for amine functions (Strader et al

(1991) 266: 5-8). These and other acidic, potential secondary interaction points for amino-substituted metal-ion chelators can be addressed through construction of a small number of metal-ion sites placed in their spatial vicinity - as described above for Asp^{171} (AspIV:20). Similarly amino-functions in a biological target molecule – for example, epsilon amino groups of Lys residues –can be addressed by, for example tetrazol substituted metal-ion chelators. Importantly, a Cys residue will be introduced at a similar appropriate distance from the engineered metal-ion site and bipyridine analogs will be synthesized with thiol reactive groups such as thiols or thiosulfonates positioned in variale distances from the bipyridine center. Through oxidation, a covalent bond will then form between the thiol reagent and the Cys in the receptor, which thereby will be irreversibly blocked in its function.

After testing and optimisation *in vitro*, the genetically modified receptors will be introduced into genetically modified test animals through various forms of standard methods, for example as follows in Example 54.

EXAMPLE 52

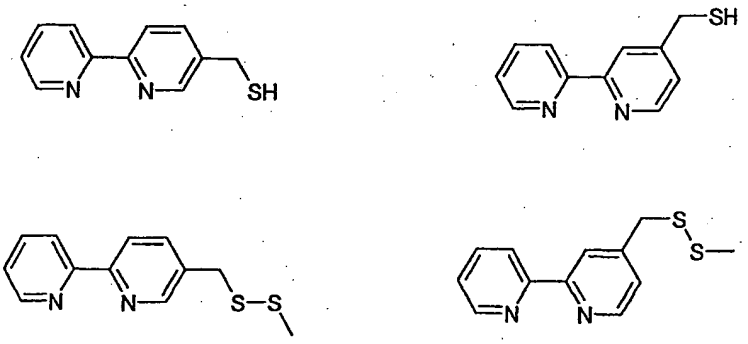
Generating covalent bonds between thiol- and disulfide-modified bipyridine analogs and natural Cys residues in biological target molecules harboring engineered anchoring metal-ion sites

Establishment of a irreversible, covalent bond between the test compound and the biological target molecule will make the target validation method more generally applicable due to the more efficient receptor inhibition which will be achieved. The leukotrien LTB4 and the bombesin BRS-3 receptors are used as example of receptors which have naturally occurring Cys residues facing the main ligand binding crevice and which accordingly can be addressed by thiol-modified metal-ion chelators from a metal-ion site engineered into the receptor in the vicinity of the Cys residue.

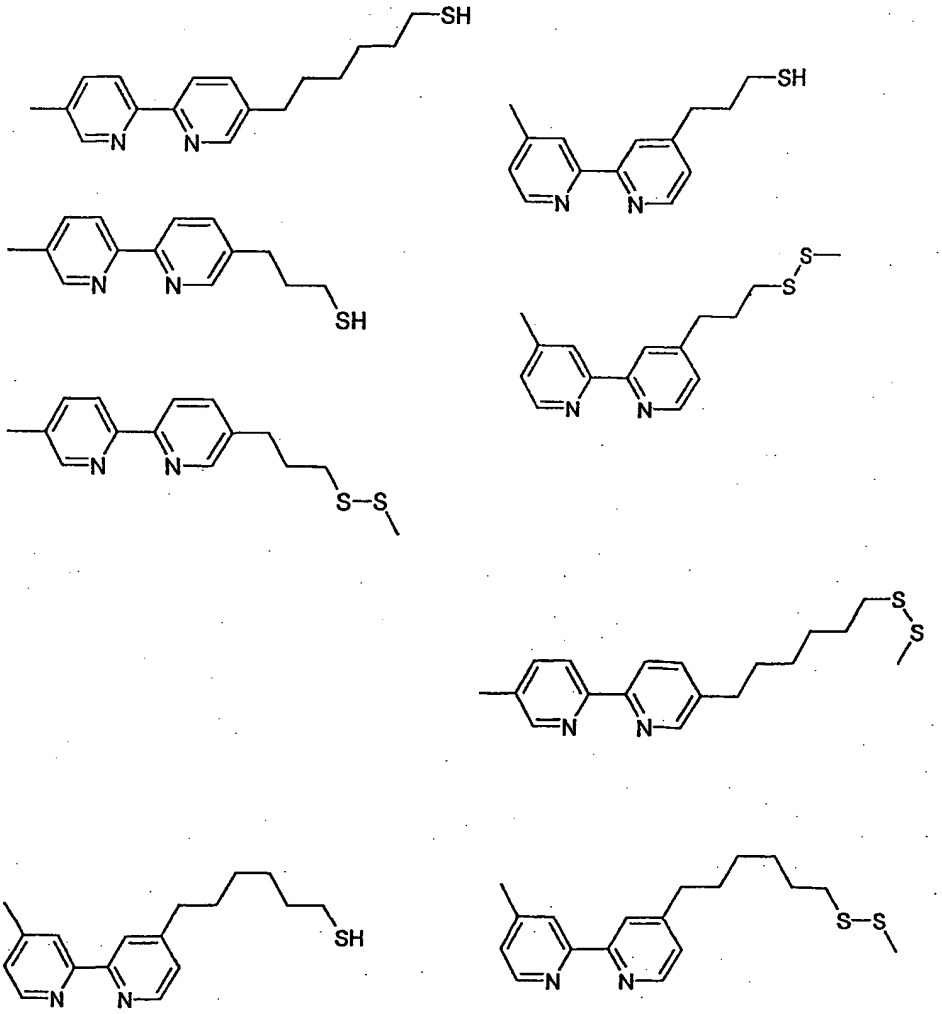
Methods – The cDNA coding for, for the leukotrien LTB4 receptor (BLT) and the bombesin BRS-3 receptor can be expressed in COS-7 cells as described for other 7TM and 12TM proteins previously. Metal-ion sites may be engineered through PCR-directed mutagenesis and the functional activity of the receptor be tested for instance by established ligand binding methods employing the radioactive ligands [^{3}H]-LTB4 for the LTB4 receptor and [125I]-[Tyr6,betaAla11,Phe13,Nle14]bombesin-6-14 for the BRS-3

receptor. For both receptors signal transduction can be measured for example by determination of intracellular calcium mobilization by classical methods.

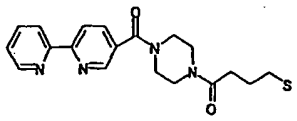
Results - Metal-ion binding sites will be introduced in the LTB4 receptor in the spatial vicinity of the "target residues" CysIII:04 and CysIII:08 by introduction of His residues at positions V:01 and V:05 and between V:05 and VI:24 as previously demonstrated in the NK1 receptor (Elling et al. EMBO J. (1996) 15: 6213-6219). Similarly, metal-ion binding sites will be introduced in the bombesin BRS-3 receptor in the spatial vicinity of the "target residue" CysV:08 by introduction of His residues at positions V:01 and V:05, between V:05 and VI:24 and between III:05 and V:01 as also previously demonstrated in the NK1 receptor. A library of thiol- and disulfide-derivatized bipyridine analogs will be synthesized including compounds as shown below:



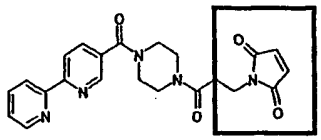
5



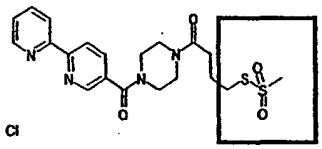
Other Examples of leaving groups/functionalities that may be advantageous when generating covalent bonds between a biological target molecule and a thiol-modifed bipyridine analog.



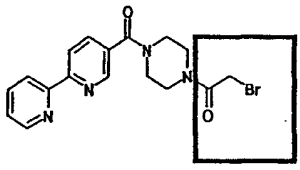
TM523



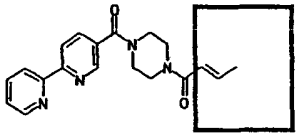
TM522



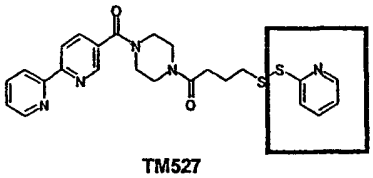
TM524



TM526



TM541



TM527

In a typical experiment, thiol- and disulfide-substituted chelators will be tested in complex with either Zn(II) or Ru(II) in the metal-ion-site engineered LTB-4 and the BRS-3 receptors, and the compounds ability to inhibit the binding of [^{125}I]-LTB-4 or the binding of [125I]-[Tyr6,betaAla11,Phe13,Nle14]bombesin-6-14 and/or the ability of the compounds to inhibit the signal transduction induced by the appropriate agonists for these receptors will be tested. Binding of the bipyridine moiety to the engineered metal-ion site will generate a local high concentration of the thiol or the disulfide, which in the present example are

placed on a non-constraining spacer arm of variable length. Due to the spatial proximity of the thiol- or disulfide-function on the bipyridine analogs and the Cys residue in the receptors a disulfide bridge will through oxidation be formed between the bipyridine analog and the receptor. It is know, that for example in the LTB4 receptor that oxidative modification of the Cys residue will block binding and function of LTB4. Thus, the covalent binding of the thiol-reactive bipyridine analogs will efficiently block receptor function. As controls, chemical reduction of the covalent bond between the bipyridine analog and the biological target molecule by for example careful treatment with DTT will regenerate a functional receptor.

EXAMPLE 53

Generating covalent bonds between thiol- and disulfide modified bipyridine analogs and an engineered Cys residue in a biological target molecules with an engineered anchoring metal-ion sites.

Establishment of a irreversible, covalent bond between the test compound and the biological target molecule will make the target validation method more generally applicable due to the more efficient receptor inhibition which will be achieved. However, in most cases free Cys residues are not available in the biological target molecule. However, since introduction of Cys residues is known to be well tolerated in, for example 7TM and 12 TM molecules, Cys residues will be introduced at key positions as targets for thiol-reactive metal-ion chelators in biological target molecules in order to render them susceptible for inhibition by thiol reactive metal-ion chelators in the target validation process and thereby make the process using covalent inhibitors a generally applicable method. In this example, it is demonstrated that this can be done in vitro in a receptor, the RASSL version of the kappa-opioid receptor, which previously has been expressed in genetically modified animals and whose function has been shown *in vivo* to be controllable by small organic, synthetic compounds acting selectively on this receptor (Redfern et al. (1999) Nat. Biotechnol. 17: 165-67). From a systematic mutational mapping project performed in the kappa opioid receptor it is know that certain residues can be substituted in the main ligand-binding crevice without affecting, for example the binding and function of non-peptide agonists employed for activation of the RASSL receptor construct (unpublished observations).

Methods – see Example 50

Results – Single Cys residues will be individually introduced at positions IV:24, IV:20, AspV:01and III:05 in the RASSL receptor harboring a silent metal-ion site engineered into positions V:05 to VI:24. In a typical experiment, a library containing thiol- and disulfide-substituted chelators as shown in example no. 3, will be tested in complex with either Zn(II) or Ru(II) in the Cys engineered / Metal-ion site engineered RASSL version of the kappa opioid receptor. That is, the compounds ability to inhibit the binding of [3H]-diprinorphine or the compounds ability to inhibit the signal transduction induced by the non-peptide opioid agonists will be tested.

Binding of the bipyridine moiety to the engineered metal-ion site will generate a local high concentration of the thiol or the disulfide, which in the present example are placed on a non-constraining spacer arm of variable length. Due to the spatial proximity of the thiol- or disulfide-function placed on the bipyridine analogs and the Cys residue, which deliberately has been engineered into the receptor in the vicinity of the silent metal-ion site, a disulfide bridge will through oxidation be formed between the bipyridine analog and the receptor. The covalent binding of the thiol-reactive bipyridine analogs will efficiently block receptor function. As controls, chemical reduction of the covalent bond between the bipyridine analog and the biological target molecule by for example careful treatment with DTT will regenerate a functional receptor, just as the inventors previously have carefully been able to open up a mutationally engineered disulfide bridge as opposed to destroying the important disulfide bridge between the extracellular end of TM-III and the middle of extracellular loop-2 (C.E.Elling et al. Biochemistry (2000) 39: 667-75).

After testing and optimization *in vitro*, the genetically modified receptors will be introduced into genetically modified test animals through various forms of standard methods, for example as follows in Example 54.

EXAMPLE 54

Introduction of genetically modified biological target molecules into genetically modified test animals

Exchange of the endogenous receptor with the metal-ion site engineered receptor, using a technique that permits expression in exactly the same place of the genome and under the same promoter. There are several different approaches to achieve this kind of knock in, but the method used here is the strategy called "double replacement". In the double replacement method a single target embryonic stem (ES) cell clone is used for generation

of a series of replacement alleles. It is performed in a way that ensures, that no selection marker or other heterologous sequence remains in the modified locus. The method includes two rounds of homologous recombination. First a positive/negative selectable marker cassette is introduced into the locus of interest by conventional replacement. After positive selection with gentamicin (in case of neo^r as positive selection marker) only the cells containing the modified locus are remaining. In a second transfection, a targeting vector spanning the same genomic region including the desired mutation, but no selection marker is used. Selection against the negative selection marker, will exclude those cell, which have achieved the selection cassette by insertion instead of by replacement in the first round.

This method permits direct exchange of the endogenous gene with the mutated gene, however a considerable proportion of the cells in the ES cell clone will be with out the replacement. The resulting animal will accordingly end up as a mosaic. Furthermore a number of mating has to be performed in order to achieve the pure mutant form of the mice.

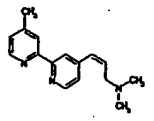
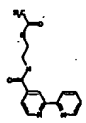
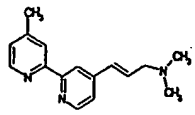
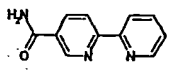
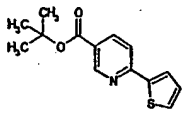
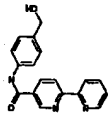
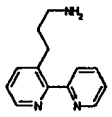
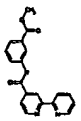
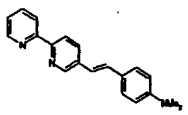
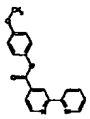
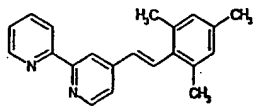
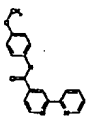
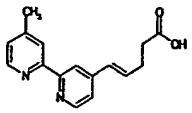
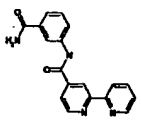
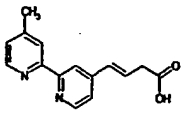
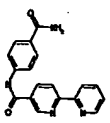
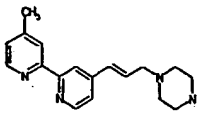
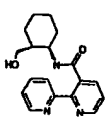
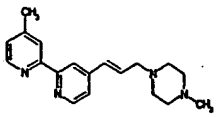
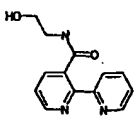
In some cases it is possible that it will be preferable to use a different approach, which permit expression of the mutant form of the receptor under a tissue specific promoter in a classical knock out animal of the relevant gene. In order to prevent the compensatory mechanism, the heterozygote knock-out animal is mated with the transgenic expressing modified receptor, and afterwards mated to achieve homozygocity of the knock out.

In the experimental section, a 7TM receptor is for convenience used as an example of a biological target molecule. In this system, very useful molecular models are available, which have been refined and have allowed for, for example the construction of intra- and especially inter-helical metal-ion sites. However, due to lack of, for example an array of suitable X-ray structures of this or similar targets in complex with agonists and antagonists it is not possible to apply classical structure-based drug design methodology in full. Nevertheless, for example in these membrane proteins the present method does to a certain degree compensate for the lack of knowledge of the detailed 3D structure of the target molecule by anchoring the lead compound and thereby creating a fix-point for the subsequent medicinal chemical optimisation point guided by the molecular models.

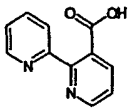
The approach described above could be further helped and guided by detailed knowledge of the 3D structure(s) of the biological target molecule, preferentially determined in complex initially with the un-substituted metal-ion chelator and subsequently in complex

with the chemically modified metal-ion chelator in which attempts have been made to establish first one secondary interaction and subsequently further secondary or tertiary interactions. For some biological target molecules such as soluble proteins this can be achieved through for example crystallization and standard X-ray analysis procedures or through, for example NMR analysis of the complex in solution again using standard procedures. Here, the method can take advantage of methods developed for structure-based drug discovery in general. This would make it possible to apply classical structure-based approaches for the establishment of secondary and tertiary interaction sites for the lead compound in the target molecule. However, it should be noted, that a major advantage and difference of the present method is, that the lead compound is anchored to a particular site and thereby to a certain degree in a particular conformation in the biological target molecule through binding to the bridging metal-ion site while the compound is being optimised for chemical recognition with the target molecule.

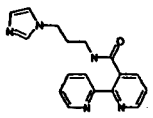
Examples of compounds used in the examples

MOLSTRUCTURE	MOL - CORPORATE ID	MOLSTRUCTURE	MOL - CORPORATE ID
	TM482		TM279
	TM483		TM241
	TM486		TM263
	TM487		TM293
	TM489		TM276
	TM492		TM276
	TM490		TM304
	TM491		TM307
	TM505		TM333
	TM506		TM334

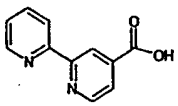
Examples of compounds used in the examples



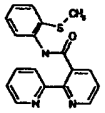
TM502



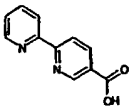
TM335



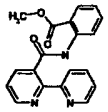
TM503



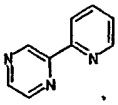
TM336



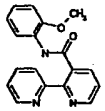
TM504



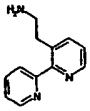
TM337



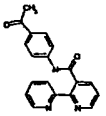
TM509



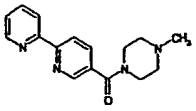
TM338



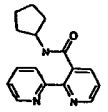
TM510



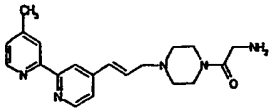
TM339



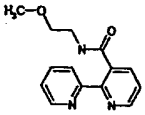
TM514



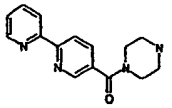
TM340



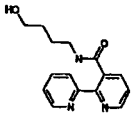
TM515



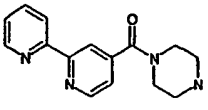
TM341



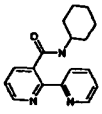
TM516



TM342

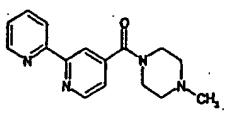


TM517

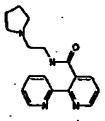


TM343

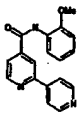
Examples of compounds used in the examples



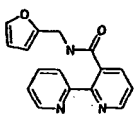
TM518



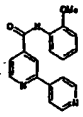
TM344



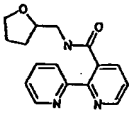
TM520



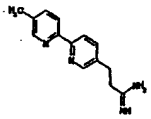
TM345



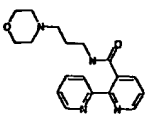
TM520



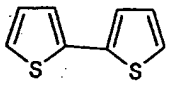
TM346



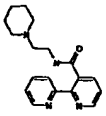
TM521



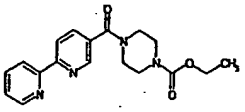
TM347



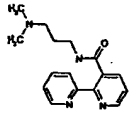
TM316



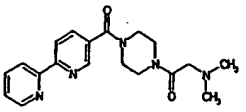
TM348



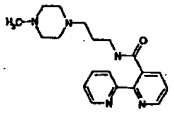
TM528



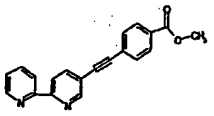
TM349



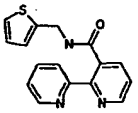
TM529



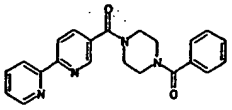
TM350



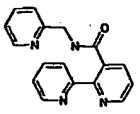
TM620



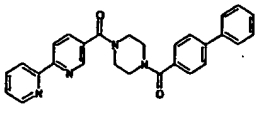
TM351



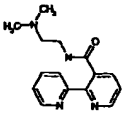
TM538



TM352



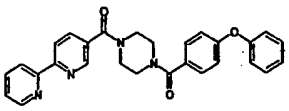
TM542



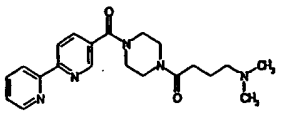
TM353

Examples of compounds used in the examples

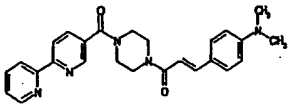
TM539



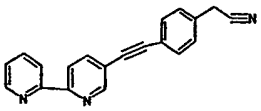
TM546



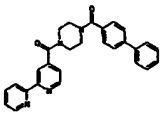
TM547



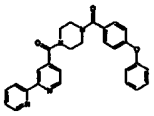
TM553



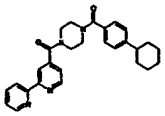
TM566



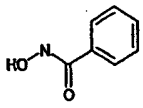
TM569



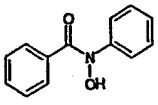
TM571



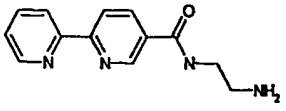
TM573



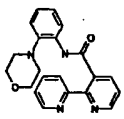
TM574



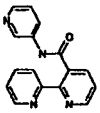
TM577



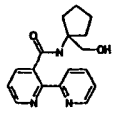
TM354



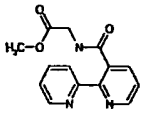
TM355



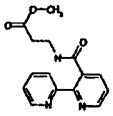
TM356



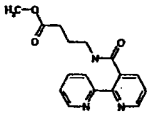
TM357



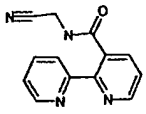
TM358



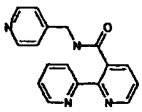
TM359



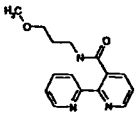
TM360



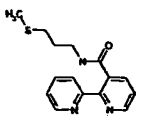
TM361



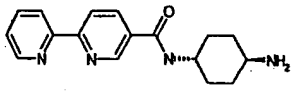
TM362



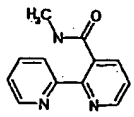
TM363



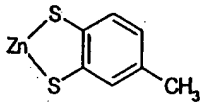
Examples of compounds used in the examples



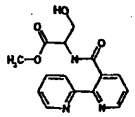
TM578



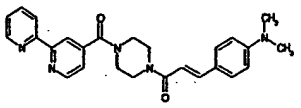
TM364



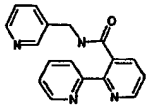
TM579



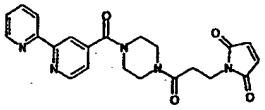
TM365



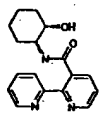
TM597



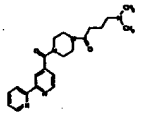
TM366



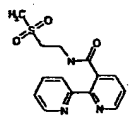
TM598



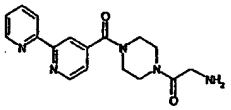
TM367



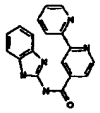
TM600



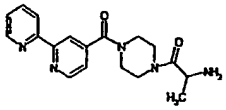
TM368



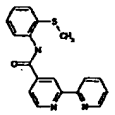
TM602



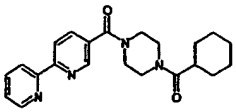
TM369



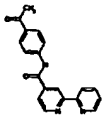
TM603



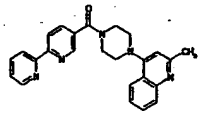
TM325



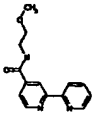
TM580



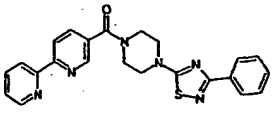
TM326



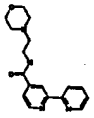
TM581



TM327

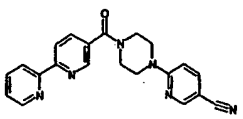


TM582

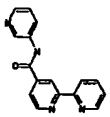


TM370

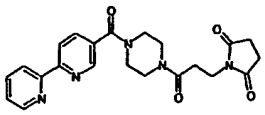
Examples of compounds used in the examples



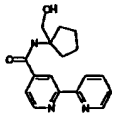
TM583



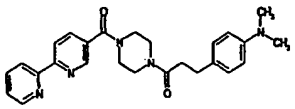
TM328



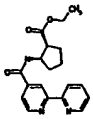
TM590



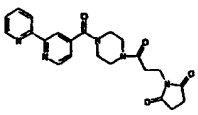
TM329



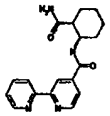
TM591



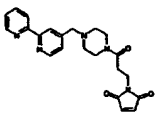
TM371



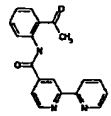
TM612



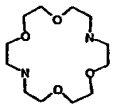
TM372



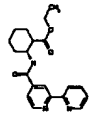
TM613



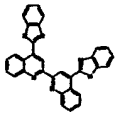
TM373



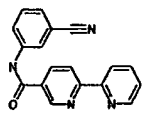
TM61



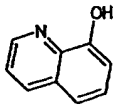
TM374



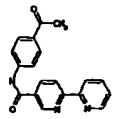
TM85



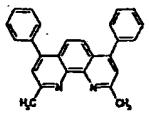
TM318



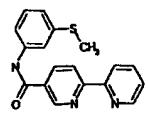
TM151



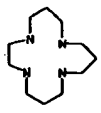
TM319



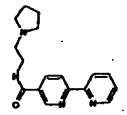
TM162



TM375

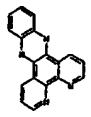


TM129

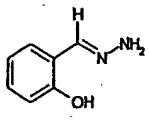


TM376

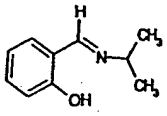
Examples of compounds used in the examples



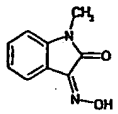
TM183



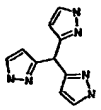
TM197



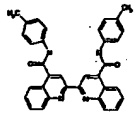
TM208



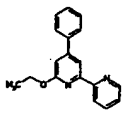
TM218



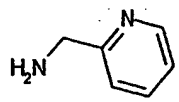
TM232



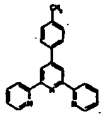
TM74



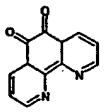
TM120



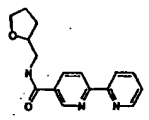
TM152



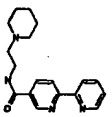
TM164



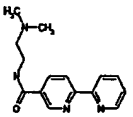
TM175



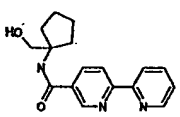
TM377



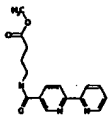
TM378



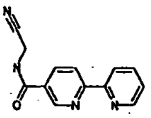
TM379



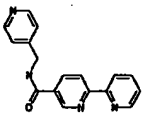
TM380



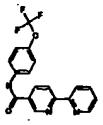
TM381



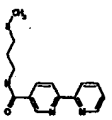
TM320



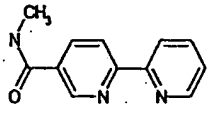
TM382



TM383



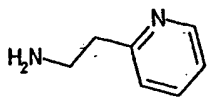
TM384



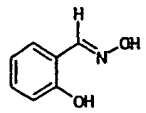
TM385

Examples of compounds used in the examples

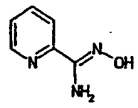
TM184



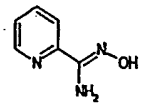
TM198



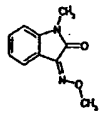
TM209



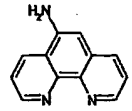
TM209



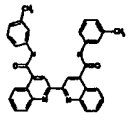
TM219



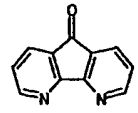
TM136



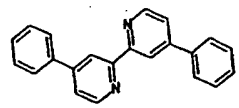
TM75



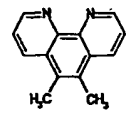
TM123



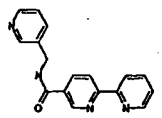
TM153



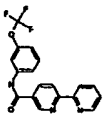
TM165



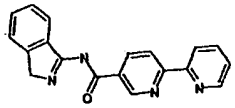
TM386



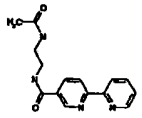
TM387



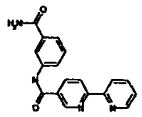
TM321



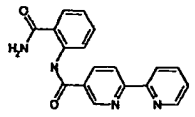
TM388



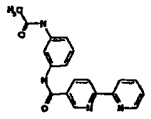
TM389



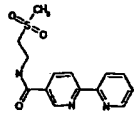
TM322



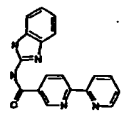
TM390



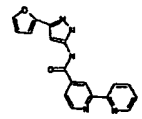
TM391



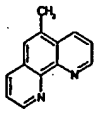
TM392



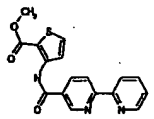
TM332



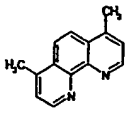
Examples of compounds used in the examples



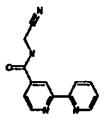
TM176



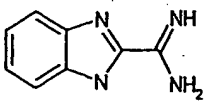
TM317



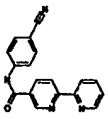
TM185



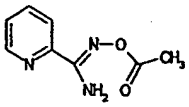
TM330



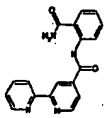
TM200



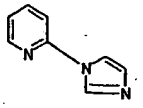
TM393



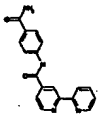
TM210



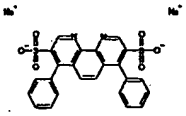
TM331



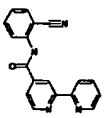
TM220



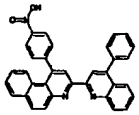
TM323



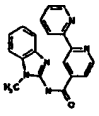
TM157



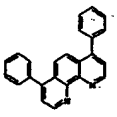
TM324



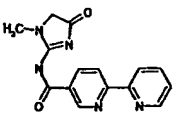
TM78



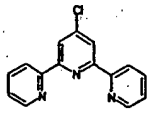
TM394



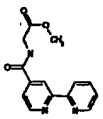
TM131



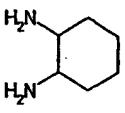
TM395



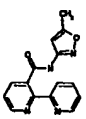
TM154



TM396

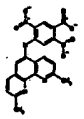


TM166

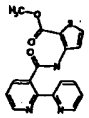


TM412

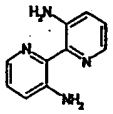
Examples of compounds used in the examples



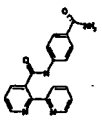
TM178



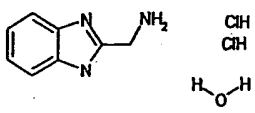
TM413



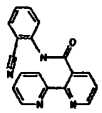
TM186



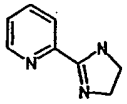
TM414



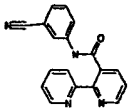
TM203



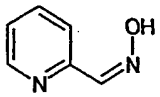
TM415



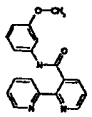
TM212



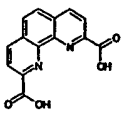
TM416



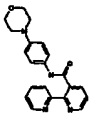
TM221



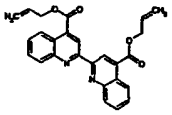
TM417



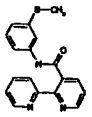
TM177



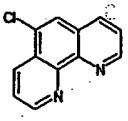
TM418



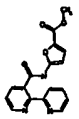
TM79



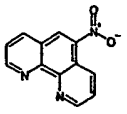
TM419



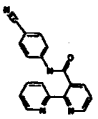
TM132



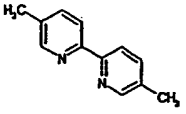
TM420



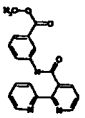
TM155



TM421

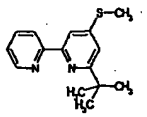


TM168

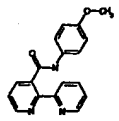


TM422

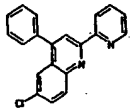
Examples of compounds used in the examples



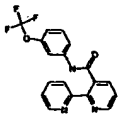
TM179



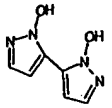
TM423



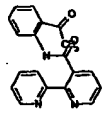
TM192



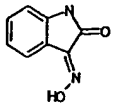
TM424



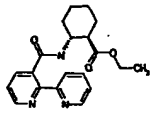
TM204



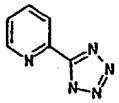
TM425



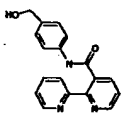
TM214



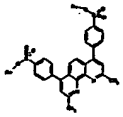
TM426



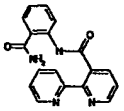
TM222



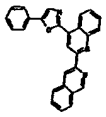
TM427



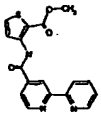
TM187



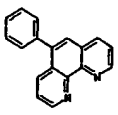
TM428



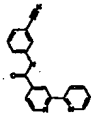
TM80



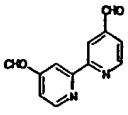
TM403



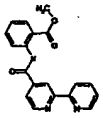
TM134



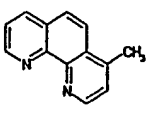
TM405



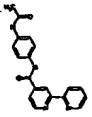
TM159



TM406

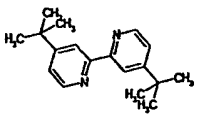


TM170

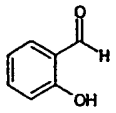


TM407

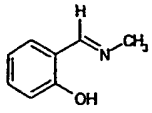
Examples of compounds used in the examples



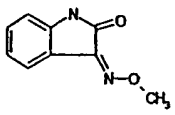
TM180



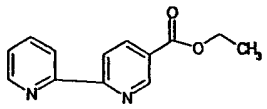
TM194



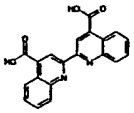
TM205



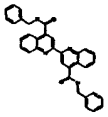
TM215



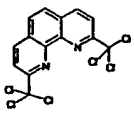
TM223



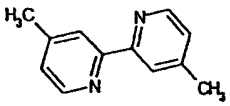
TM188



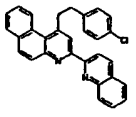
TM81



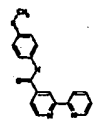
TM135



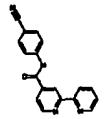
TM160



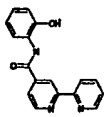
TM171



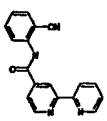
TM408



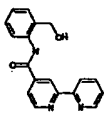
TM409



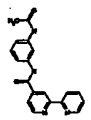
TM429



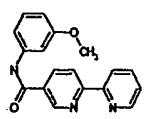
TM429



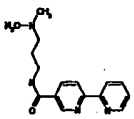
TM430



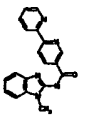
TM410



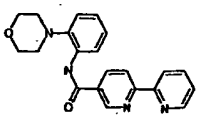
TM400



TM431

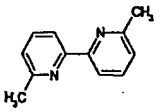


TM401

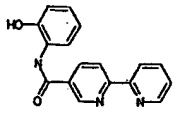


TM432

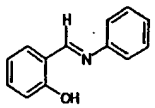
Examples of compounds used in the examples



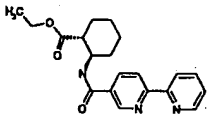
TM181



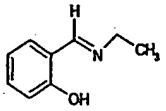
TM402



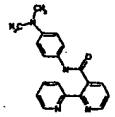
TM195



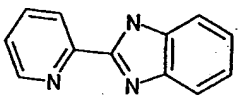
TM433



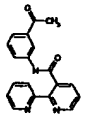
TM206



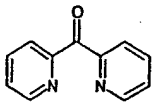
TM434



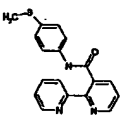
TM216



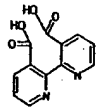
TM435



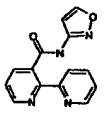
TM230



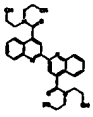
TM436



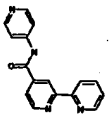
TM189



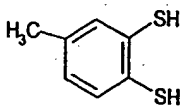
TM437



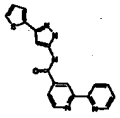
TM83



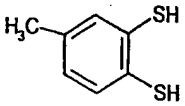
TM404



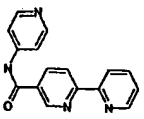
TM137



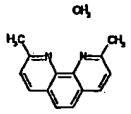
TM411



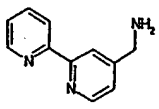
TM137



TM399



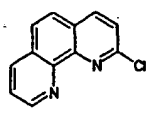
TM161



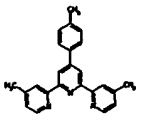
TM465

Examples of compounds used in the examples

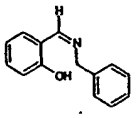
TM173



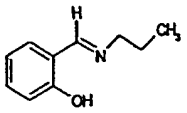
TM182



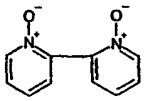
TM196



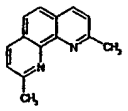
TM207



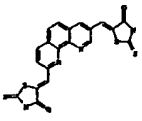
TM217



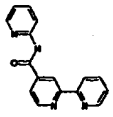
TM231



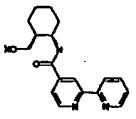
TM191



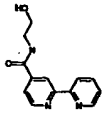
TM310



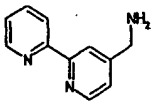
TM264



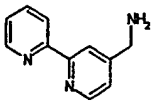
TM283



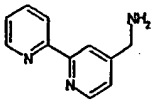
TM465



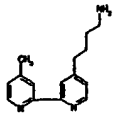
TM465



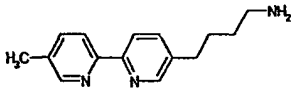
TM465



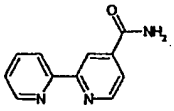
TM440



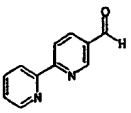
TM460



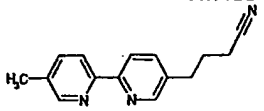
TM438



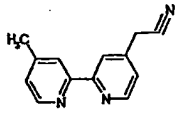
TM456



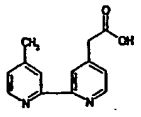
TM459



TM464

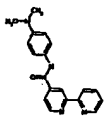


TM477

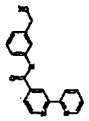


Examples of compounds used in the examples

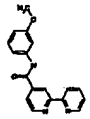
TM265



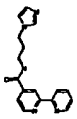
TM266



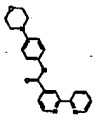
TM284



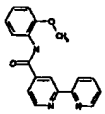
TM267



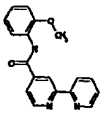
TM268



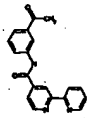
TM269



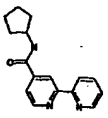
TM269



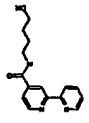
TM270



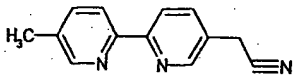
TM285



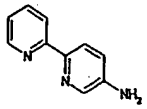
TM286



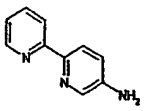
TM466



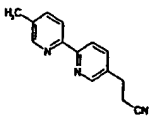
TM398



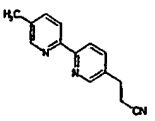
TM398



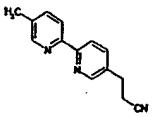
TM461



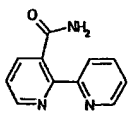
TM461



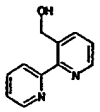
TM461



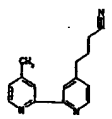
TM439



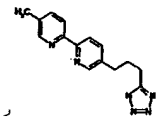
TM455



TM444

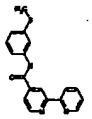


TM473

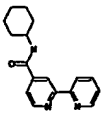


Examples of compounds used in the examples

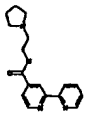
TM271



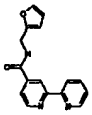
TM311



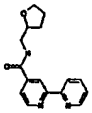
TM312



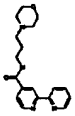
TM313



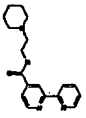
TM287



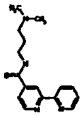
TM273



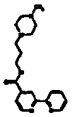
TM274



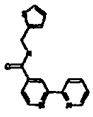
TM275



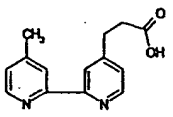
TM288



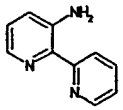
TM289



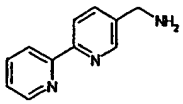
TM478



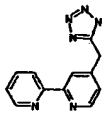
TM458



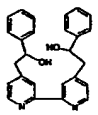
TM462



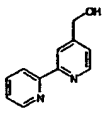
TM472



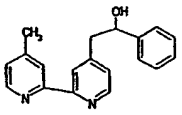
TM442



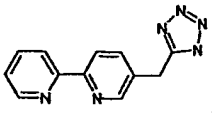
TM453



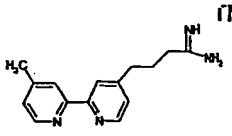
TM441



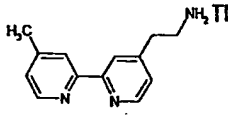
TM475



TM479

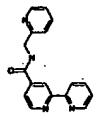


TM467

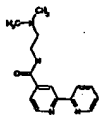


Examples of compounds used in the examples

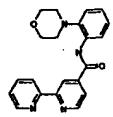
TM290



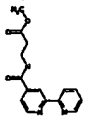
TM291



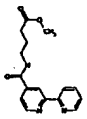
TM292



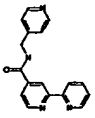
TM294



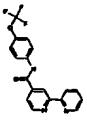
TM295



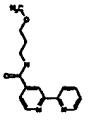
TM296



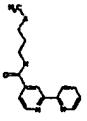
TM297



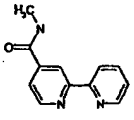
TM298



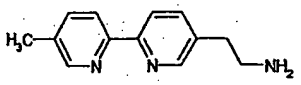
TM299



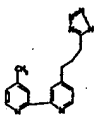
TM300



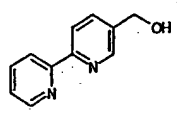
TM468



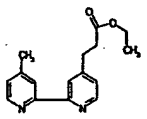
TM447



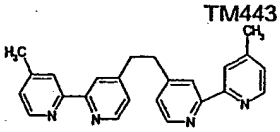
TM454



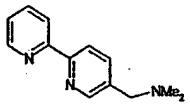
TM448



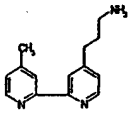
TM443



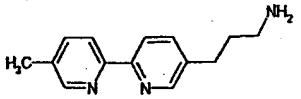
TM480



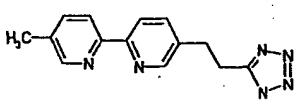
TM446



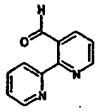
TM469



TM474

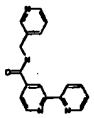


TM457

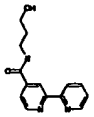


Examples of compounds used in the examples

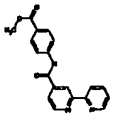
TM301



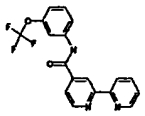
TM302



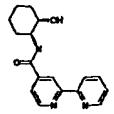
TM277



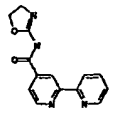
TM278



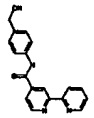
TM303



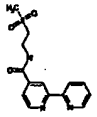
TM280



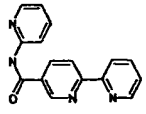
TM281



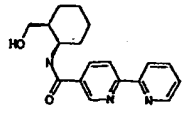
TM282



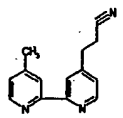
TM305



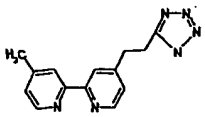
TM306



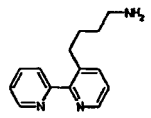
TM445



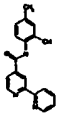
TM463



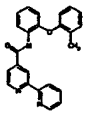
TM485



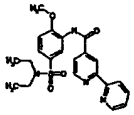
TM703



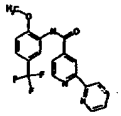
TM711



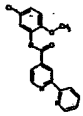
TM719



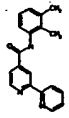
TM727



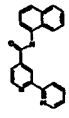
TM735



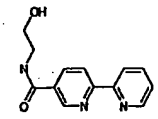
TM704



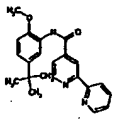
TM712



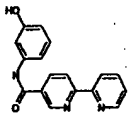
Examples of compounds used in the examples



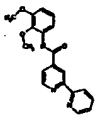
TM236



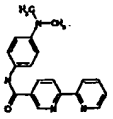
TM720



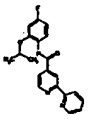
TM237



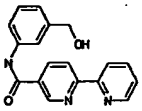
TM728



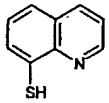
TM314



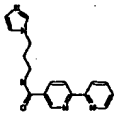
TM736



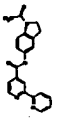
TM238



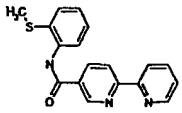
TM694



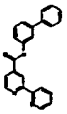
TM308



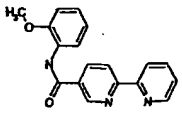
TM705



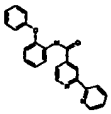
TM239



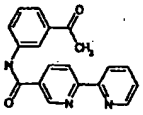
TM713



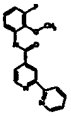
TM240



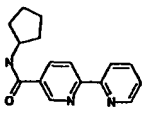
TM721



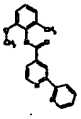
TM242



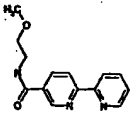
TM729



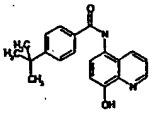
TM243



TM737

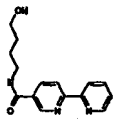


TM244

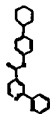


TM674

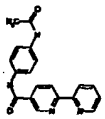
Examples of compounds used in the examples



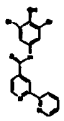
TM245



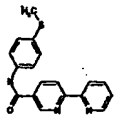
TM706



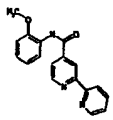
TM246



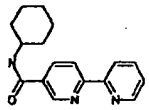
TM714



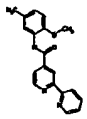
TM247



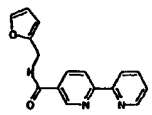
TM722



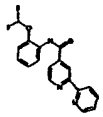
TM315



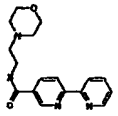
TM730



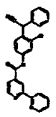
TM248



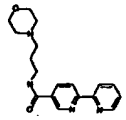
TM738



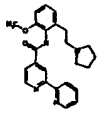
TM249



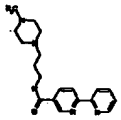
TM707



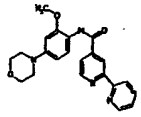
TM250



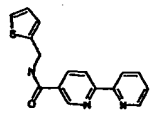
TM715



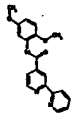
TM251



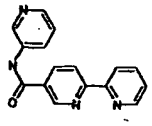
TM723



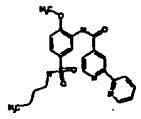
TM252



TM731

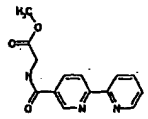


TM253

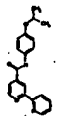


TM739

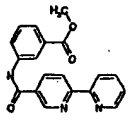
Examples of compounds used in the examples



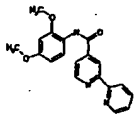
TM254



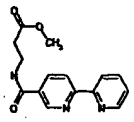
TM708



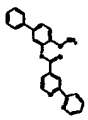
TM255



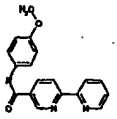
TM716



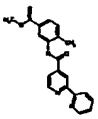
TM256



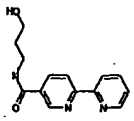
TM724



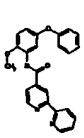
TM257



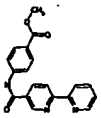
TM732



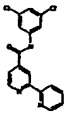
TM258



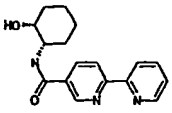
TM740



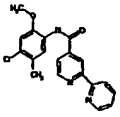
TM259



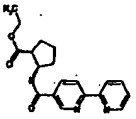
TM709



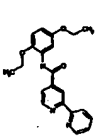
TM309



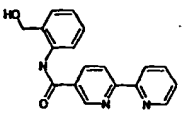
TM717



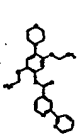
TM260



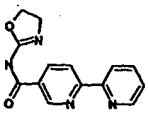
TM725



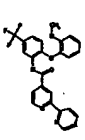
TM261



TM733

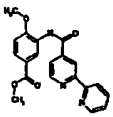


TM262

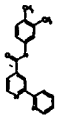


TM741

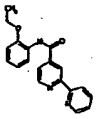
Examples of compounds used in the examples



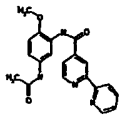
TM272



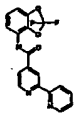
TM710



TM734



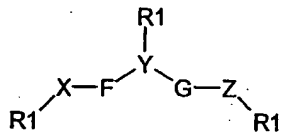
TM718



TM779

CLAIMS

1. Use of a test compound or a library comprising test compounds of the general formula I, abbreviated as Che-R^1,



Formula I

wherein F is N, O, S, Se or P; and G is N, O, S, Se or P;

X, Y and Z, which are the same or different, are straight or branched C_1-C_{12} alkyl, C_1-C_{12} alkenyl, C_1-C_{12} alkynyl, C_1-C_{12} cyclyl, aryl, C_1-C_{12} heteroalkyl, C_1-C_{12} heteroalkenyl, C_1-C_{12} heteroalkynyl, C_1-C_{12} heterocyclyl, heteroaryl;

R^1 may be present anywhere on the X, Y and/or Z moiety and it may be present on X, Y and/or Z up to as many times as possible, i.e. if X is $-CH_2-CH_2-$, then R^1 may be present on the first and/or second carbon atom one or several times; R^1 may optionally be hydrogen;

X may together with Y and/or Z fuse to form a cyclic ring system;
Y may together with X and/or Z fuse to form a cyclic ring system;
X, Y and Z may together fuse to form a cyclic ring system;

R^1 corresponds to a structure –A-B-C, wherein the element A is a coupling or connecting moiety, B is a spacer moiety and C is a functional group; –B- may be substituted one or more times with a further C, which may be the same or different, and

A linked to be –A-B-C is selected from the group consisting of:

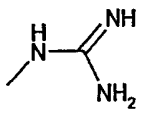
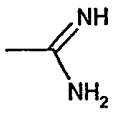
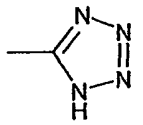
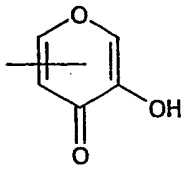
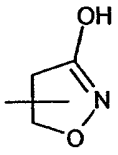
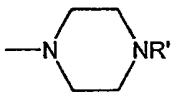
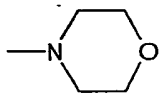
-O-, -S-, -NH-, -N=, -N<, $-CH_2-$, -C(=O)-, $-PO_3-$, $-PO_2NH-$, $-NHPO_2$, -NHP(O)<, -C≡C-, -CH=CH-, -SO-, $-SO_2-$, -COO-, -CONR"-, -NR'CO-, $-NR'SO_2-$, $-SO_2NR"-$, -CH(OH)-, -CR'(OH)-, -CR'(O-alk)-, -N-alk-, aryl, cycloalkyl, heteroaryl, heterocycloalkyl etc., and the term "alk" includes straight or branched alkyl, straight or branched alkenyl and straight or branched alkynyl; R' is H or lower alk, i.e. C_1-C_6; R" is as defined below;

-B- is absent or selected from the group consisting of:

H, alkyl, straight or branched alkyl, alkenyl (straight or branched), alkynyl (straight or branched), aryl, cycloalkyl, heteroaryl, heterocycloalkyl, alkyloxyalkyl, alkylaminoalkyl,

-C is absent or selected from the group consisting of:

-H, -OH, -NR"R"', -CONR"R"', -COOH, -COOR", -OCOR", -COR", -SO_2NR"R"', -SH, -S-S-alk, -NHCOR", -NR"COR"', $NHSO_2$R", -$NHCONH_2$, -NH-CN, -F, -Cl, -Br, -I; -SCF_3, -CF_3, -OCF_3, -SCH_3, -SR", -CN, -$N(CN)_2$,-NO_2, -OCH_3, -OR', -NH_2, -NHMe, -NHAlk, -NMe_2, -$N(Alk)_2$, -$N(Alk)_3^+$, heteroaryl, heterocycloalkyl, -$PO(OH)NH_2$, -SO_2OH,

and R" and/or R"' has the same meaning as given for B above optionally substituted with one or more C;

in those cases where a compound has two or more R^1 in positions adjacent to each other the –A- and/or –B- elements from the two individual R^1 may form a cyclic ring system;

in those cases where B is absent R^1 is –A-C or –A and in those cases where C is absent R^1 is –A-B or –A;

in some cases, A may be absent and then $-R^1$ is –B-C or –C, and B may be substituted one or more times with C, which may be the same or different;

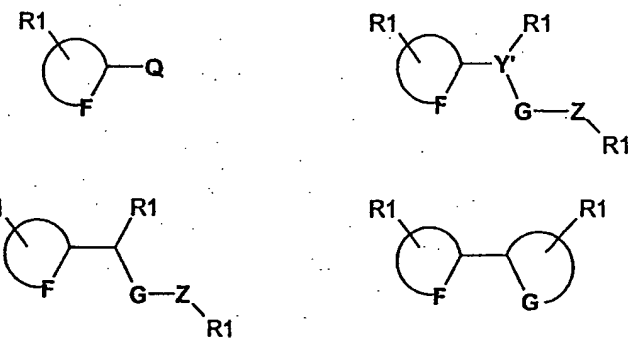
the total number of atoms (X+F+Y+G+Z) excluding hydrogen atoms is at the most 25;

the total number of heteroatoms in (X+F+Y+G+Z) is at the most 6; and

the size of a ring is at the most 14 atoms, preferably 5 or 6 atoms,

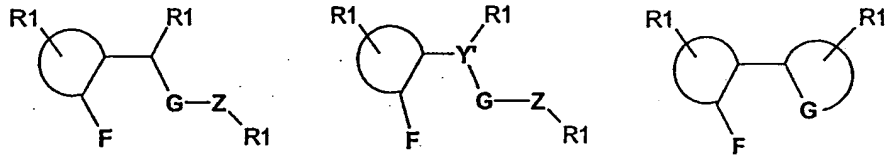
in a target validation process as described herein.

2. Use according to claim 1, wherein the compounds have the following structures



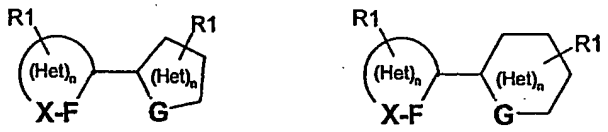
wherein Q is a structural element containing a heteroatom, Y' is the remainder of the group Y or it is absent, and F, G, Z and R^1 are as defined in formula I.

3. Use according to claim 1, wherein the compounds have one of the following structures



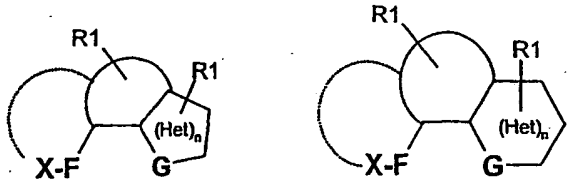
wherein F, G, Z, Y' and R1 are as defined in claims 1 or 2.

4. Use according to claim 1, wherein the compounds have one of the following structures



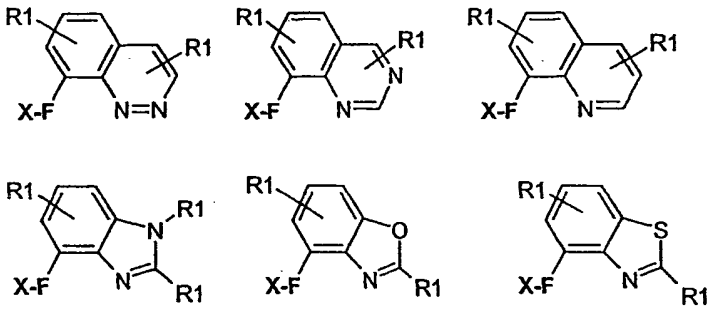
wherein the coordinating atom G is included in a 5-or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and four heteroatoms and the coordinating atom F contained within an aromatic, unsaturated or saturated 5- or 6-memebered heterocycle containing between one and four heteroatoms.

5. Use according to claim 1, wherein the compounds have one of the following structures



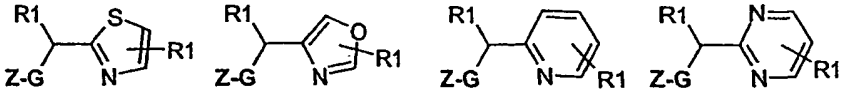
wherein the coordinating atom G is included in a 5- or 6-membered aromatic, unsaturated or saturated heterocycle containing between one and three heteroatoms and the coordinating atom F appended to an annelated aromatic, unsaturated or saturated 5- or 6-membered ring, and X-F can optionally be included in a fused ring as indicated by the dashed line.

6. Use according to claim 1, wherein the compounds have one of the following structures

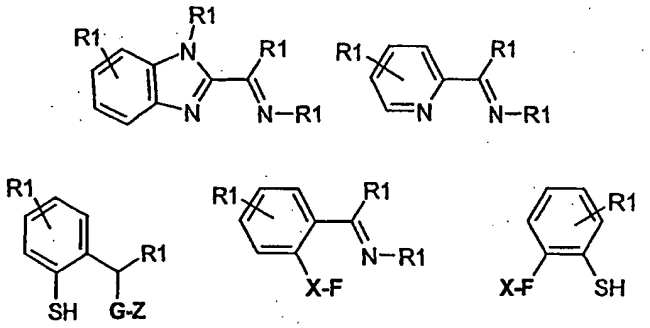


wherein R1 may be present on the annelated ring system to give mono-, di-, tri-, tetra-, pentasubstituted derivatives.

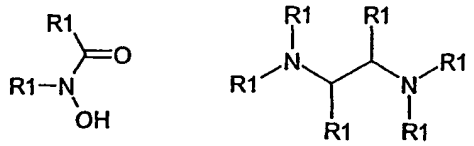
7. Use according to any of claims 1-3, wherein the compounds have one of the following structures



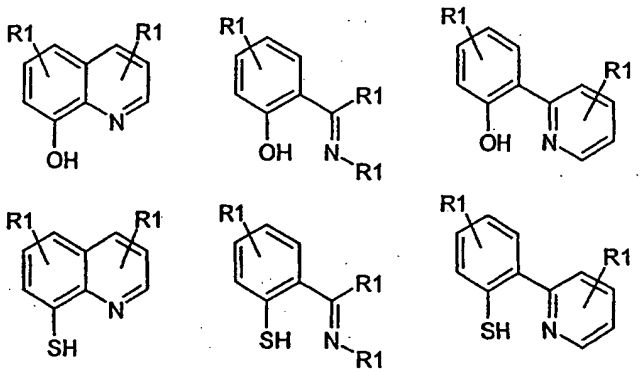
8. Use according to any of claims 1-3, wherein the compounds have one of the following structures



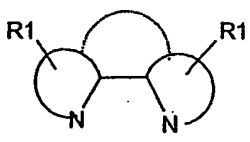
9. Use according to any of claims 1-3, wherein the compounds have one of the following structures



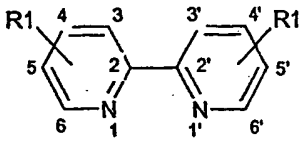
10. Use according to any of claims 1-3, wherein the compounds have one of the following structures



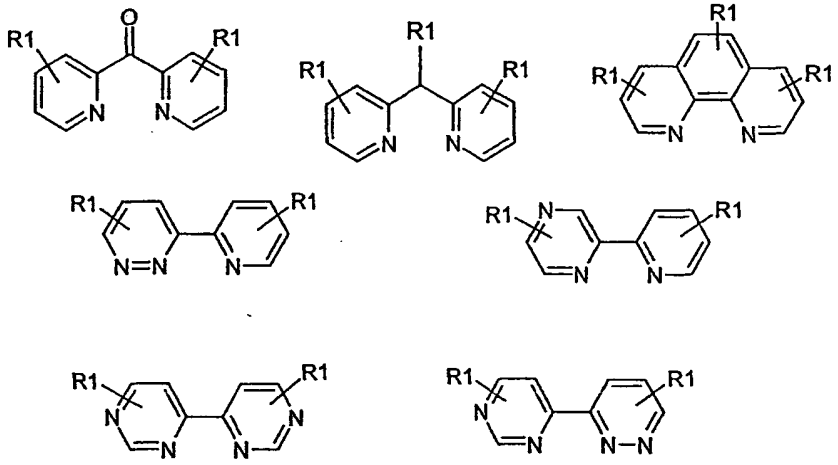
11. Use according to any of claims 1-3, wherein the compounds have one of the following structures



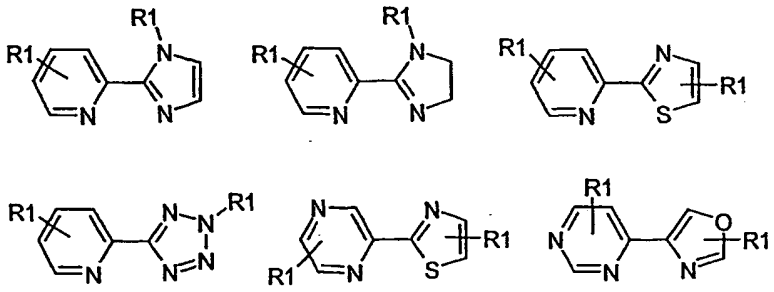
12. Use according to any of claims 1-3, wherein the compounds have one of the following structures



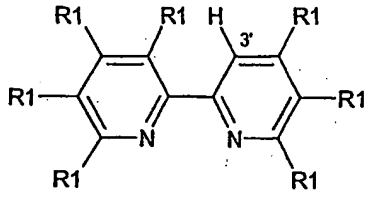
13. Use according to any of claims 1-3, wherein the compounds have one of the following structures



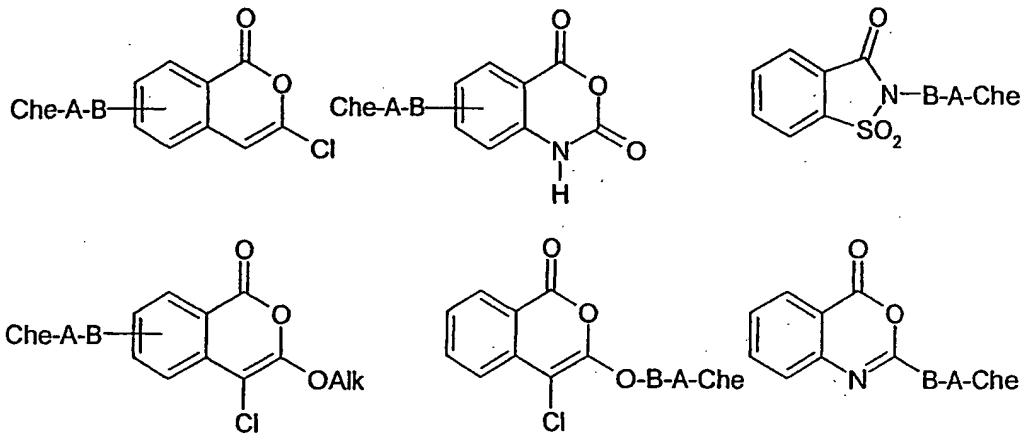
14. Use according to any of claims 1-3, wherein the compounds have one of the following structures



15. Use according to any of claims 1-3, wherein the compounds have one of the following structures



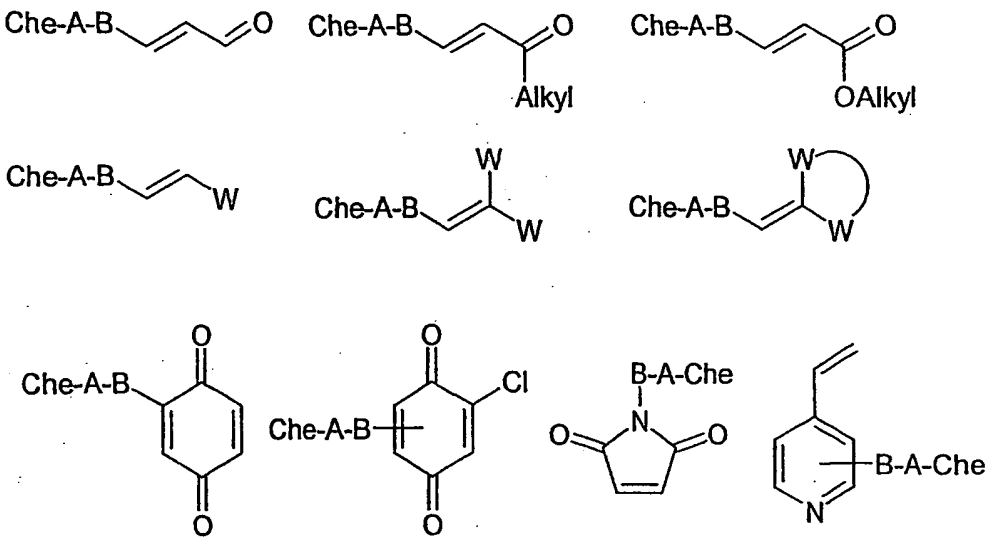
16. Use according to claim 1-15, wherein the test compound is capable to react with –SH, -OH and $–NH_2$ groups in the vicinity of the metal-ion binding site and has one of the following structures:



Che-A-B-CO-O-Alkyl Che-A-B-CO-O-Aryl Che-A-B-CO-Lg

and wherein Che represents a chelating scaffolds according to claims 1- 15, and A and B have the same meaning as in Formula I.

17. Use according to claim 1-15, wherein the test compound is capable to act as Michael acceptor for –SH, -OH and $–NH_2$ groups in the vicinity of the metal-ion binding site and has one of the following structures:



and wherein Che represents a chelating scaffolds according to claims 1- 15, and A and B have the same meaning as in Formula I.

18. Use according to claim 1-15, wherein the test compound has one of the following structures:

Che-A-B-NH-CO-CH_2-Lg

Che-A-B-CO-CH_2-Lg

Che-A-B-CH_2-Lg

and wherein Che represents a chelating scaffolds according to claims 1- 15, and A and B have the same meaning as in Formula I and wherein Lg is Br, Cl, F or other suitable leaving groups, for forming a covalent bond with a reactive functional group such as –SH, -OH, or NH_2 in the vicinity of the metal-ion binding site.

19. Use according to claim 1-15, wherein the test compound has one of the following structures:

Che-A-B-$B(OH)_2$

Che-A-B-CO-CF_3

Che-A-B-CHO

Che-A-B-C=N-Alkyl

and wherein Che represents a chelating scaffolds according to claims 1- 15 and A and B have the same meaning as in Formula I.

20. Use according to claim 1-15, wherein the test compound has one of the following structures:

Che-A-B-SH

Che-A-B-S-S-Alkyl Che-A-B-S-S-Aryl Che-A-B-S-S-Heteroaryl

Che-A-B-S-SO_2-Alkyl

Che-A-B-S-SO_3^-

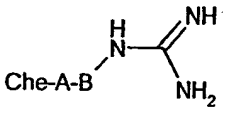
and wherein Che represents a chelating scaffolds according to claims 1- 15 and A and B have the same meaning as in Formula I.

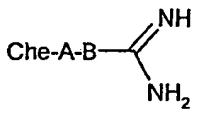
21. Use according to claim 1-15, wherein the test compound has one of the following structures:

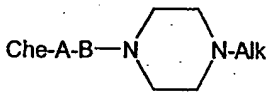
Che-A-B-NH_2

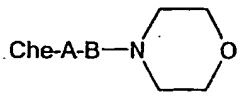
Che-A-B-NH-Alk

Che-A-B-N(Alk)$_2$









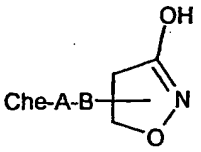
and wherein Che represents a chelating scaffolds according to claims 1- 15 and A, B and Alk have the same meaning as in Formula I.

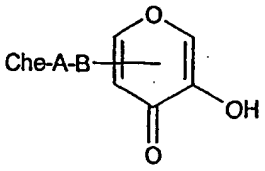
22. Use according to claim 1-15, wherein the test compound has one of the following structures or a pro-drug thereof:

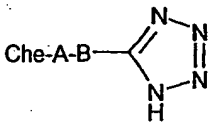
Che-A-B-COOH

Che-A-B-SO_2OH

Che-A-B-PO_3OH



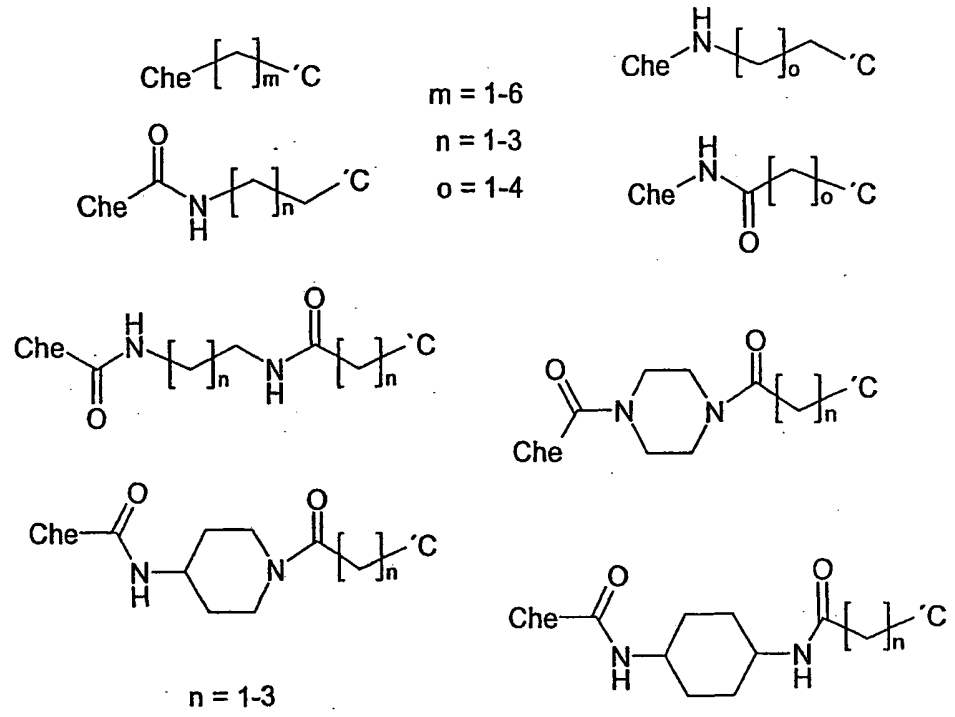




and wherein Che represents a chelating scaffolds according to claims 1- 15 and A and B have the same meaning as in Formula I.

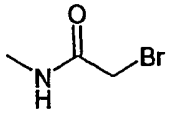
23. Use according to claim 1-15, wherein the test compound has one of the following

structures:

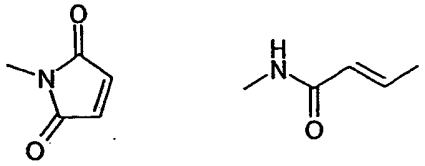


wherein alkyl chains also can be unsaturated when chemically feasible and wherein Che represents a chelating scaffolds according to claims 1- 15, and C being as defined in Formula I.

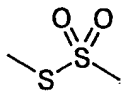
24. Use according to claim 23 wherein C being selected from alkylating and acylating groups according to claims 16 or 18 including

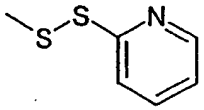


25. Use according to claim 23 wherein C being selected from slowly reversible binding groups according to claims 17 or 19 including

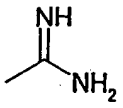
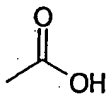
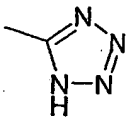


26. Use according to claim 23 wherein C being selected from thiol-scavenging groups according to claim 20 including

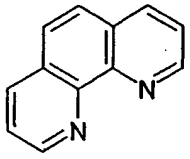
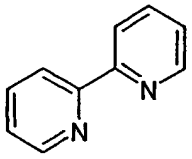
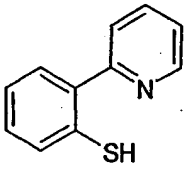
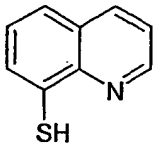
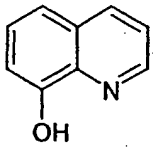




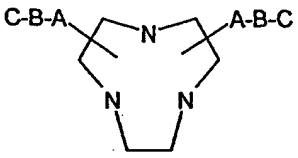
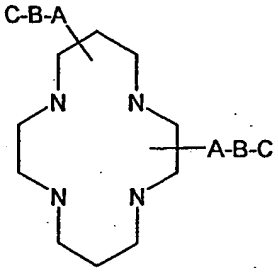
27. Use according to claim 23 wherein C being selected from charged groups according to claims 21 or 22 including

28. Use according to claims 23-27, wherein Che being selected from the chelating scaffolds:

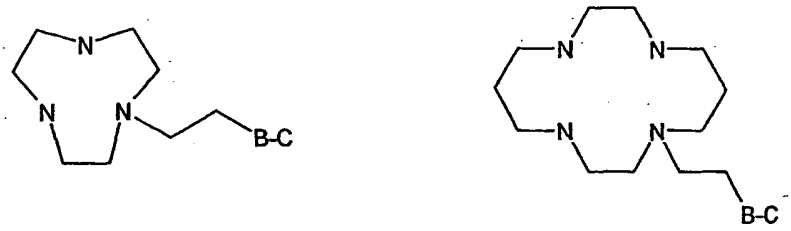
29. Use according to claim 1, wherein the test compounds have the following structures

wherein A, B, C have the same meaning as in Formula I.

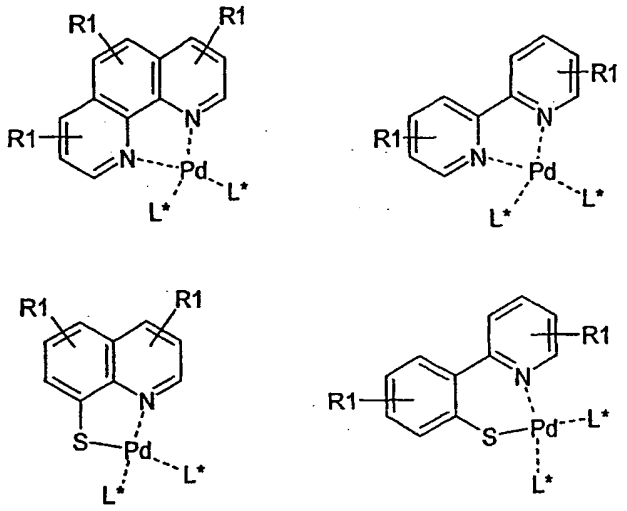
30. Use according to claims 23-27, wherein Che being selected from the chelating

scaffolds:



wherein B and C have the same meaning as in Formula I.

31. Use according to claim 1, wherein the test compounds have the following structures



and Pd may be replaced with Ru, Rh and Pt.

32. Use according to claims 23-27, wherein Che being chelated in a complex with a strongly bound metal ion according to claim 31.

33. Use according to any of the preceding claims, wherein the test compound is in a form of a complex or forms a complex with a metal ion.

34. Use according to claim 33, wherein the test compound substantially immediately before use is complexed with a metal ion.

35. Use according to claim 33 or 34, wherein the metal ion is selected from the group consisting of aluminium, antimony, arsenic, astatine, barium, beryllium, bismuth, boron,

cadmium, calcium, cerium, cesium, chromium, cobalt, copper, dysprosium, erbium, europium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, iron, lanthanum, lead, lutetium, magnesium, manganese, mercury, molybdenum, neodymium, nickel, niobium, osmium, palladium, platinum, polonium, praseodymium, promethium, rhenium, rhodium, rubidium, ruthenium, samarium, scandium, selenium, silicon, silver, strontium, tantalum, technetium, tellurium, terbium, thallium, thorium, thulium, tin, titanium, tungsten, vanadium, ytterbium, yttrium, zinc, zirconium, and oxidation states and isotopes thereof; in particular aluminium, antimony, barium, bismuth, calcium, chromium, cobalt, copper, europium, gadolinium, gallium, germanium, gold, indium, iron, lutetium, manganese, magnesium, nickel, osmium, palladium, platinum, rhenium, rhodium, rubidium, ruthenium, samarium, silver, strontium, technetium, terbium, thallium, thorium, tin, yttrium, zinc, and oxidation states or isotopes thereof; in particular calcium, cobalt, copper, europium, iron, magnesium, manganese, nickel, palladium, platinum, ruthenium, samarium, terbium and zink (and oxidation states or isotopes thereof, preferably cobalt (II, III), copper (I, II), nickel (II, III), zink (II) and platinum (0, II, V), palladium (0, II, IV), ruthenium (0, II, III, IV, VI, VIII) or isotopes thereof.

36. A target validation process according to any of items 1-50 herein, wherein an amino acid residue defining the metal-ion binding site is in a position selected from the group consisting of

Single Positions:

transmembrane segment-I:

[I:01]
[I:03]
[I:04]
[I:06]
[I:07]
[I:10]
[I:11]
[I:14]
[I:17]
[I:18]
[I:21]
[I:22]

or in positions

transmembrane segment-II:
[II:05]
[II:06]
[II:07]
[II:09]
[II:10]
[II:13]
[II:14]
[II:17]
[II:18]
[II:20]
[II:21]
[II:22]
[II:24]
[II:25]
[II:26]

or in positions

transmembrane segment-III:
[III:04]
[III:05]
[III:08]
[III:09]
[III:11]
[III:12]
[III:13]
[III:15]
[III:16]
[III:17]
[III:18]
[III:19]
[III:20]
[III:21]
[III:22]
[III:23]
[III:24]
[III:25]
[III:26]

[III:27]
[III:28]
[III:29]
[III:30]
[III:32]

or in positions

transmembrane segment-IV:
[IV:01]
[IV:02]
[IV:05]
[IV:06]
[IV:09]
[IV:10]
[IV:12]
[IV:13]
[IV:14]
[IV:16]
[IV:17]
[IV:18]
[IV:19]
[IV:20]

or in positions

transmembrane segment-V:
[V:01]
[V:04]
[V:05]
[V:08]
[V:09]
[V:12]
[V:13]
[V:16]
[V:17]
[V:20]
[V:21]
[V:23]
[V:24]
[V:27]

[V:28]

or in positions

transmembrane segment-VI:

[VI:-06]
[VI:-04]
[VI:-03]
[VI:-02]
[VI:-01]
[VI:01]
[VI:02]
[VI:03]
[VI:05]
[VI:06]
[VI:08]
[VI:09]
[VI:12]
[VI:13]
[VI:16]
[VI:17]
[VI:19]
[VI:20]
[VI:21]
[VI:23]
[VI:24]

or in positions

transmembrane segment-VII:

[VII:02]
[VII:03]
[VII:05]
[VII:06]
[VII:07]
[VII:08]
[VII:09]
[VII:10]
[VII:11]
[VII:12]
[VII:13]

[VII:15]
[VII:16]
[VII:17]
[VII:19]
[VII:20]

37. A target validation process according to claim 36, wherein an amino acid residue defining the metal-ion binding site is in a position selected from the group consisting of

TM-I:
[I:03]
[I:07]
[I:10]
[I:14]

TM-II:
[II:17]
[II:18]
[II:20]
[II:21]
[II:24]
[II:25]

TM-III:
[III:04]
[III:05]
[III:08]
[III:09]
[III:19]
[III:23]
[III:27]
[III:29]
[III:32]

TM-IV:
[IV:12]
[IV:13]

[IV:16]
[IV:17]
[IV:19]
[IV:20]

TM-V:

[V:01]
[V:04]
[V:05]
[V:08]
[V:09]
[V:12]

TM-VI:

[VI:-01]
[VI:-02]
[VI:09]
[VI:16]
[VI:19]
[VI:20]
[VI:23]
[VI:24]

TM-VII:

[VII:02]
[VII:03]
[VII:06]
[VII:07]
[VII:10]
[VII:16]

38. A target validation process according to claim 36, wherein two of the amino acid residues defining the metal-ion binding site are in a position selected from the group consisting of

transmembrane segment-I:
[I:03;I:07]

[I:06;I:10]
[I:10;I:14]
[I:14;I:18]
[I:17;I:21]
[I:18;I:22]

or in positions

transmembrane segment-II:
[II:05;II:09]
[II:06;II:10]
[II:09;II:13]
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

or in positions

transmembrane segment-III:
[III:04;III:08]
[III:05;III:09]
[III:08;III:12]
[III:09;III:13]
[III:11;III:15]
[III:12;III:16]
[III:15;III:19]
[III:19;III:23]
[III:20;III:24]
[III:21;III:25]
[III:22;III:26]
[III:23;III:27]
[III:24;III:28]

or in positions

transmembrane segment-IV:
[IV:01;IV:05]
[IV:02;IV:06]
[IV:05;IV:09]
[IV:06;IV:10]
[IV:12;IV:16]
[IV:13;IV:17]

[IV:14;IV:18]

or in positions

transmembrane segment-V:

[V:01;V:05]
[V:04;V:08]
[V:05;V:09]
[V:08;V:12]
[V:09;V:13]
[V:12;V:16]
[V:13;V:17]
[V:16;V:20]
[V:17;V:21]
[V:20;V:24]
[V:23;V:27]
[V:24;V:28]

or in positions

transmembrane segment-VI:

[VI:-04;VI:01]
[VI:-03;VI:02]
[VI:-02;VI:03]
[VI:01;VI:05]
[VI:02;VI:06]
[VI:05;VI:09]
[VI:08;VI:12]
[VI:09;VI:13]
[VI:13;VI:17]
[VI:16;VI:20]
[VI:17;VI:21]
[VI:19;VI:23]
[VI:20;VI:24]

or in positions

transmembrane segment-VII:

[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]
[VII:07;VII:11]
[VII:09;VII:13]

[VII:12;VII:16]
[VII:13;VII:17]
[VII:15;VII:19]
[VII:16;VII:20]

or in positions

transmembrane segment-I and -II
[I:01;II:26]
[I:01;II:25]
[I:01;II:22]
[I:03;II:25]
[I:04;II:26]
[I:04;II:25]
[I:04;II:22]
[I:07;II:25]
[I:07;II:22]
[I:07;II:21]
[I:07;II:18]
[I:10;II:18]
[I:10;II:14]
[I:10;II:13]
[I:11;II:18]
[I:11;II:14]
[I:14;II:18]
[I:14;II:13]
[I:14;II:14]
[I:14;II:10]
[I:18;II:10]
[I:18;II:07]

or in positions

transmembrane segment-I and -VII
[I:03;VII:07]
[I:03;VII:03]
[I:07;VII:07]

or in positions

transmembrane segment-II and -III
[II:20;III:04]

[II:24;III:04]

or in positions

transmembrane segment-II and -VII

[II:17;VII:10]

[II:18;VII:10]

[II:21;VII:07]

or in positions

transmembrane segment-III and -IV

[III:05;IV:17]

[III:05;IV:20]

[III:09;IV:16]

[III:09;IV:17]

or in positions

transmembrane segment-III and –V

[III:05;V:01]

[III:05;V:04]

[III:05;V:08]

[III:09;V:01]

[III:09;V:04]

[III:09;V:08]

[III:12;V:08]

[III:13;V:08]

[III:13;V:12]

[III:16;V:12]

[III:16;V:16]

[III:17;V:16]

[III:19;V:16]

[III:20;V:16]

[III:23;V:27]

[III:23;V:23]

[III:26;V:20]

[III:26;V:24]

[III:26;V:27]

[III:27;V:23]

[III:27;V:27]

[III:30;V:23]

[III:30;V:27]

or in positions

transmembrane segment-III and -VI
[III:05;VI:16]
[III:08;VI:16]
[III:08;VI:13]
[III:09;VI:13]
[III:12;VI:13]
[III:13;VI:09]
[III:16;VI:09]
[III:19;VI:09]
[III:19;VI:06]
[III:19;VI:05]
[III:19;VI:09]
[III:22;VI:05]
[III:23;VI:09]
[III:25;VI:02]
[III:25;VI:-03]
[III:26;VI:02]
[III:26;VI:-03]
[III:26;VI:-02]
[III:26;VI:-06]
[III:27;VI:-01]
[III:27;VI:-02]
[III:29;VI:-02]
[III:30;VI:-06]
[III:30;VI:-02]
[III:32;VI:-02]

or in positions

transmembrane segment-III and -VII
[III:04;VII:07]
[III:05;VII:06]
[III:09;VII:09]
[III:08;VII:06]
[III:08;VII:10]
[III:11;VII:10]

[III:12;VII:06]
[III:12;VII:10]
[III:12;VII:12]
[III:15;VII:16]
[III:19;VII:16]

or in positions

transmembrane segment-IV and -V
[IV;19;V:04]
[IV;20;V:04]
[IV;12;V:12]
[IV;16;V:08]

or in positions

transmembrane segment-V and -VI
[V:01;VI:20]
[V:01;VI:24]
[V:05;VI:20]
[V:05;VI:24]
[V:09;VI:20]
[V:09;VI:21]
[V:09;VI:17]

or in positions

transmembrane segment-VI and -VII
[VI:23;VII:02]
[VI:23;VII:05]
[VI:20;VII:02]
[VI:19;VII:05]
[VI:19;VII:02]
[VI:16;VII:02]
[VI:16;VII:06]
[VI:16;VII:09]
[VI:16;VII:10]
[VI:12;VII:08]
[VI:12;VII:09]
[VI:09;VII:12]
[VI:09;VII:15]
[VI:05;VII:16]

[VI:05;VII:15]

39. A target validation process according to any of claims 36-38, wherein two of the amino acid residues defining the metal-ion binding site is in a position selected from the group consisting of

TM-I:
[I:03;I:07]
[I:10;I:14]

TM-II:
[II:17;II:21]
[II:20;II:24]
[II:21;II:25]

TM-III:
[III:04;III:08]
[III:05;III:09]

TM-IV:
[IV:12;IV:16]
[IV:13;IV:17]

TM-V:
[V:01;V:05]
[V:04;V:08]
[V:05;V:09]

TM-VI:
[VI:16;VI:20]
[VI:19;VI:23]
[VI:20;VI:24]

TM-VII:
[VII:02;VII:06]
[VII:03;VII:07]
[VII:06;VII:10]

TM-I / TM-VII

[I:03;VII:07]

[I:07;VII:07]

[I:03;VII:03]

TM-II / TM-III

[II:20;III:04]

[II:24;III:04]

TM-II / TM-VII

[II:17;VII:10]

[II:18;VII:10]

[II:21;VII:07]

TM-III / TM-IV

[III:05;IV:17]

[III:05;IV:20]

[III:09;IV:16]

[III:09;IV:17]

TM-III / TM-V

[III:05;V:01]

TM-III / TM-VI

[III:08;VI:16]

[III:19;VI:09]

[III:23;VI:09]

[III:27;VI:-01]

[III:27;VI:-02]

[III:29;VI:-02]

[III:32;VI:-02]

TM-III / TM-VII

[III:08;VII:06]

[III:19;VII:16]

TM-IV / TM-V

[IV;19;V:04]

[IV;20;V:04]

[IV;12;V:12]

[IV;16;V:08]

TM-V / TM-VI

[V:01;VI:20]

[V:01;VI:24]

[V:05;VI:20]

[V:05;VI:24]

TM-VI / TM-VII

[VI:23;VII:02]

[VI:23;VII:06]

[VI:19;VII:02]

[VI:19;VII:06]

40. A target validation process according to any of the preceding claims 36-39, wherein the amino acid in any of the positions claimed is selected from the group consisting of His, Cys, Asp, Glu, Trp, Tyr, Ser, Thr, Lys, Arg, Asn, Gln and Met.

41. A target validation process according to any of claims 36-39, wherein the amino acid in any of the positions claimed is selected from the group consisting of His, Cys, Asp and Glu.

42. A target validation discovery process according claim 38 or 39 wherein the amino acids in any of the positions claimed is selected from the group consisting of

His-His

His-Cys

His-Asp

His-Glu

Cys-Cys

Cys-His

Cys-Asp

Cys-Glu

Asp-Asp
Asp-His
Asp-Cys
Asp-Glu
Glu-Glu
Glu-Asp
Glu-His
Glu-Cys

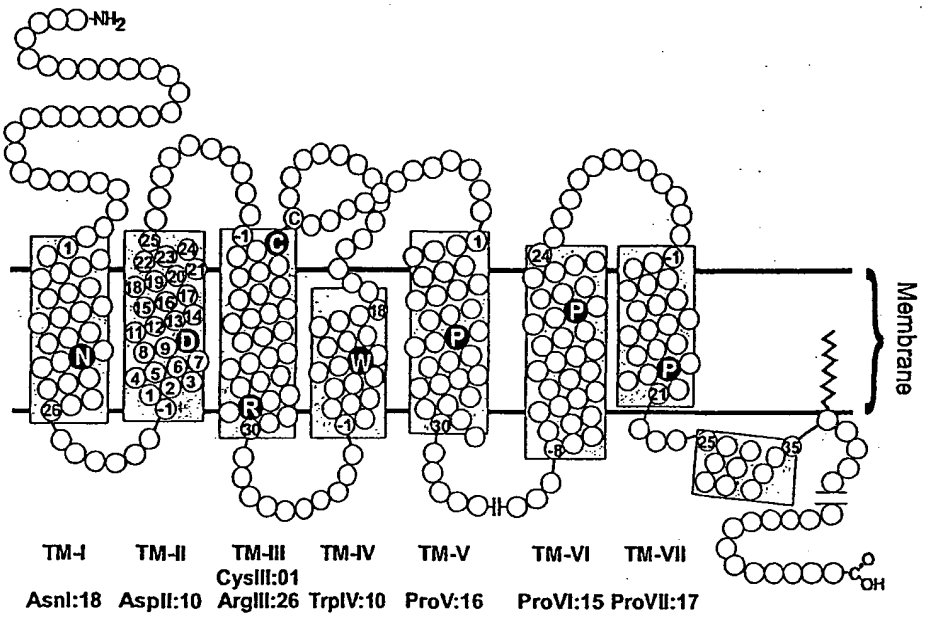

NH2
Membrane
TM-I
TM-II
TM-III
TM-IV
TM-V
TM-VI
TM-VII
CysIII:01
AsnI:18
AspII:10
ArgIII:26
TrpIV:10
ProV:16
ProVI:15
ProVII:17
OH

Fig. 1

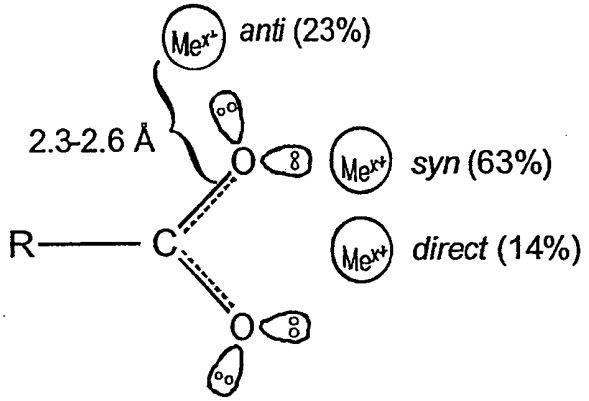


Fig. 2

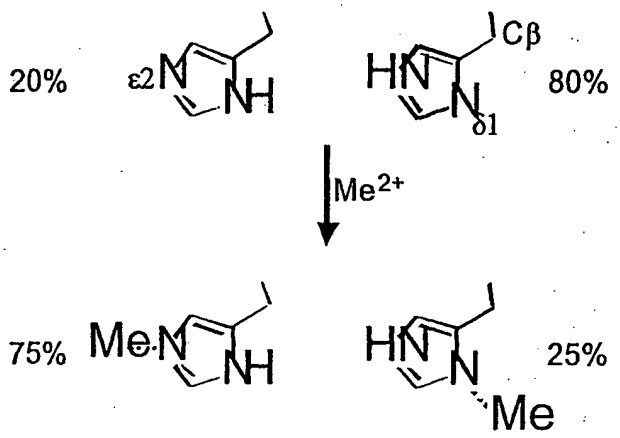


Fig. 3

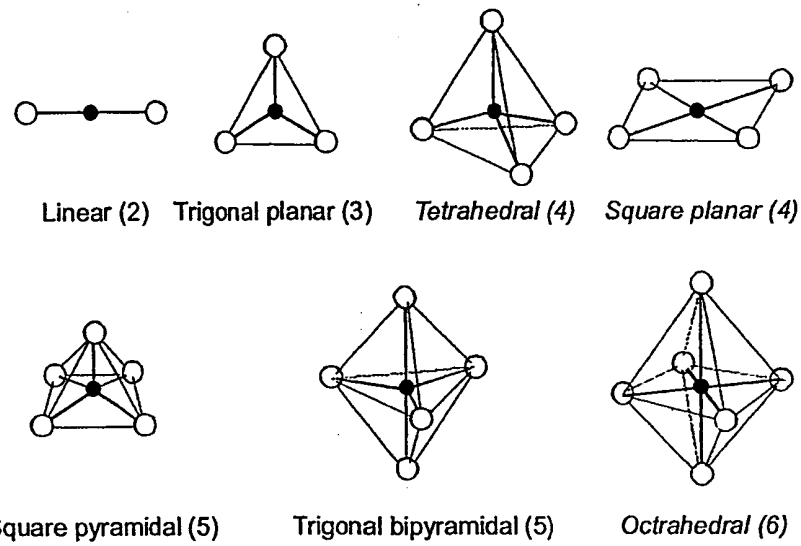

Linear (2)
Trigonal planar (3)
Tetrahedral (4)
Square planar (4)
Square pyramidal (5)
Trigonal bipyramidal (5)
Octrahedral (6)

Fig. 4

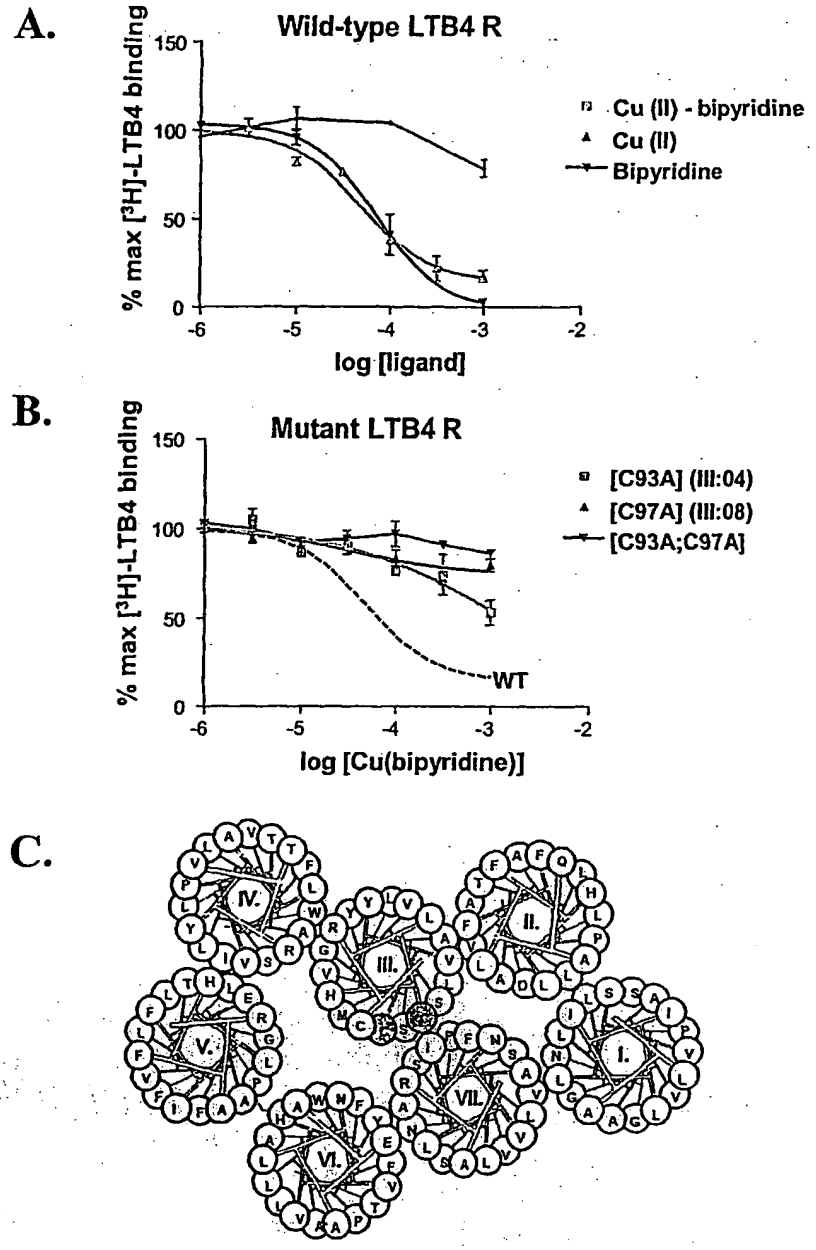
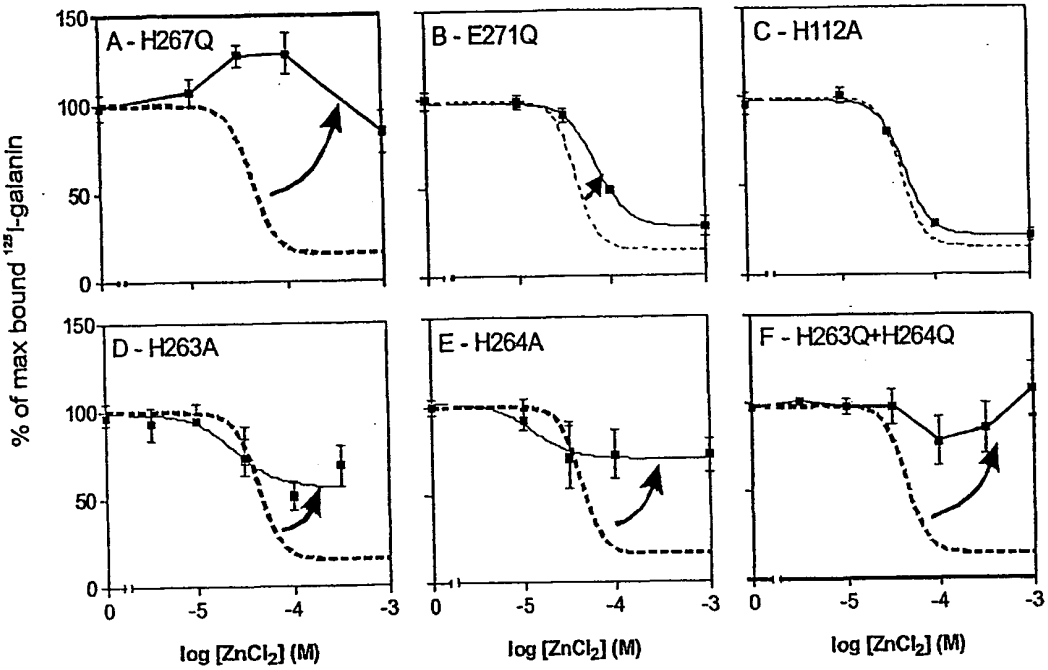

A.
Wild-type LTB4 R
% max [3H]-LTB4 binding
150
100
50
0
-6
-5
-4
-3
-2
log [ligand]
Cu (II) - bipyridine
Cu (II)
Bipyridine
B.
Mutant LTB4 R
% max [3H]-LTB4 binding
log [Cu(bipyridine)]
[C93A] (III:04)
[C97A] (III:08)
[C93A;C97A]
WT
C.
I.
II.
III.
IV.
V.
VI.
VII.

Fig. 5



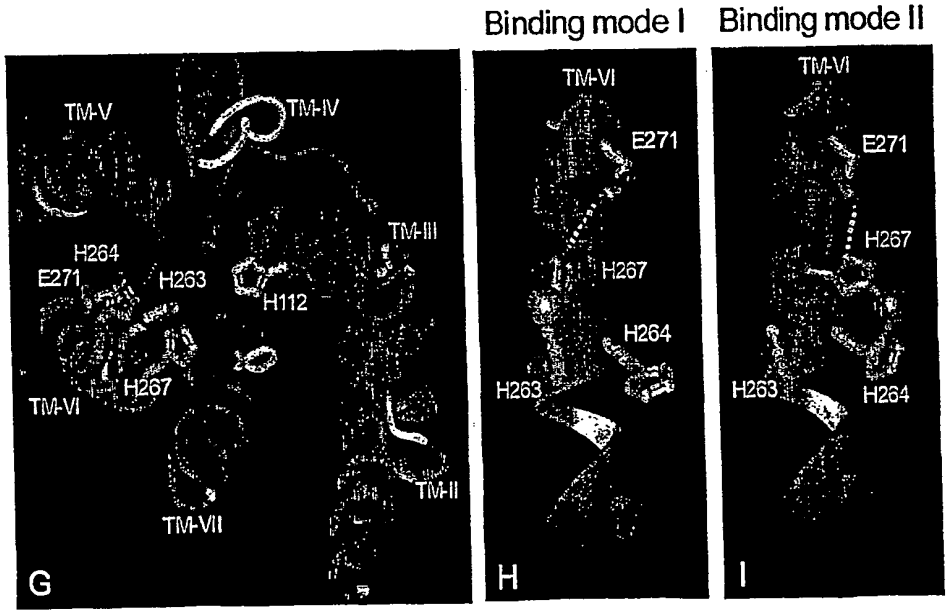
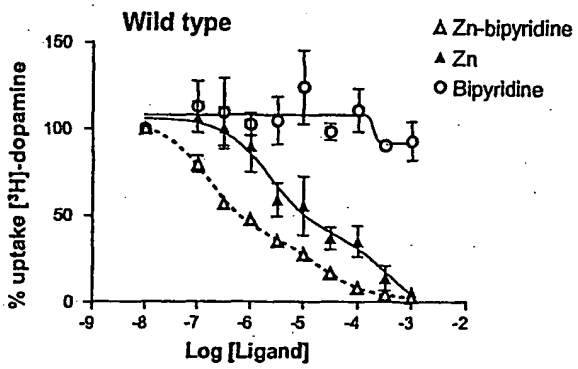
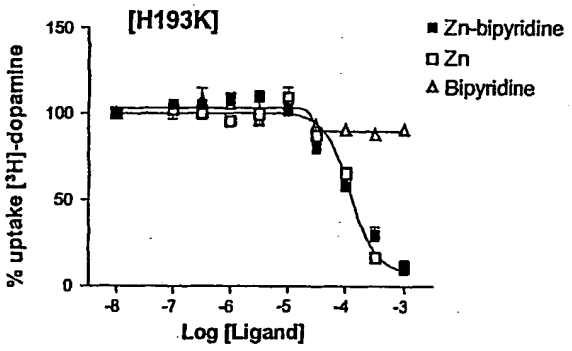


Fig. 6

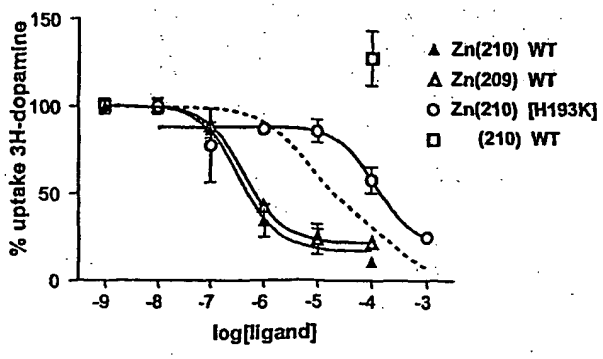
A.


Wild type
Zn-bipyridine
Zn
Bipyridine
% uptake [³H]-dopamine
Log [Ligand]

B.


[H193K]
Zn-bipyridine
Zn
Bipyridine
% uptake [³H]-dopamine
Log [Ligand]

C.


Zn(210) WT
Zn(209) WT
Zn(210) [H193K]
(210) WT
% uptake 3H-dopamine
log[ligand]

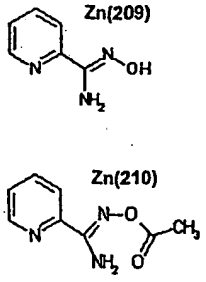
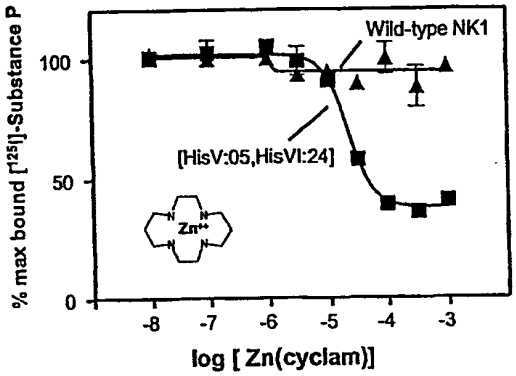

Zn(209)
Zn(210)

Fig. 7

A.

		Ions				1,10-Phenanthroline-complexes				2,2-Bipyridine-complexes			
		Zn	±SEM	(n)	Cu ±SEM (n)	Zn	±SEM	(n)	Cu ±SEM (n)	Zn	±SEM	(n)	Cu ±SEM (n)
WTHNK1		320	±20	(5)	370±30 (5)	490	±50	(4)	480±60 (3)	390	±60	(4)	150±30 (2)
Y92H	II:24;III:04	17	± 3	(3)	28± 5 (2)	26	± 5	(2)	28± 6 (3)	31	± 5	(3)	25± 3 (2)
E193H;N109H	III:05;V:01	13	± 5	(2)	120±20 (2)	46	± 9	(2)	120±20 (2)	13	± 4	(2)	160±30 (2)
P112H;M291C	III:08;VII:06	41	± 9	(6)	82±15 (2)	63	± 4	(5)	45±12 (4)	21	± 2	(4)	13± 2 (4)
Y272H	V:05;VI:24	9.1	± 1	(3)	330±50 (3)	6.8	± 1.2	(3)	150±20 (2)	9.8	± 3.2	(2)	140±20 (2)

B.



C.

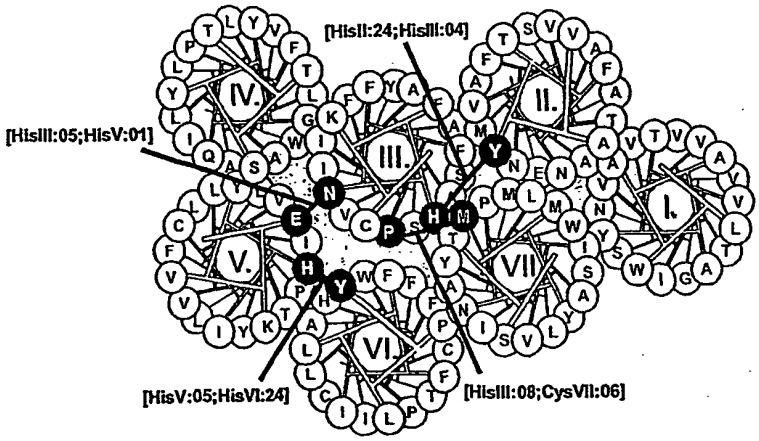


Fig. 8

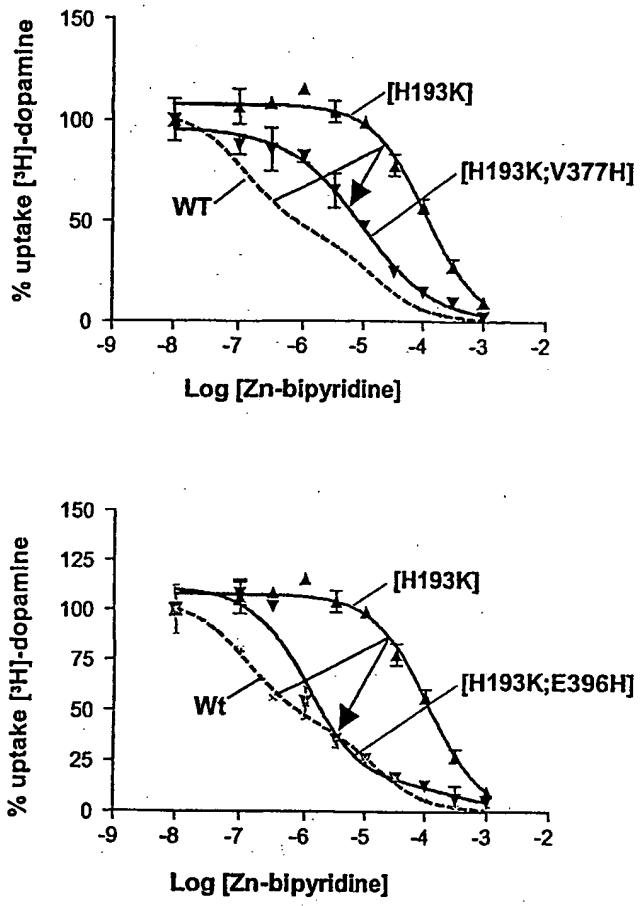

150
100
50
0
% uptake [³H]-dopamine
[H193K]
[H193K;V377H]
WT
-9
-8
-7
-6
-5
-4
-3
-2
Log [Zn-bipyridine]
150
125
100
75
50
25
0
% uptake [³H]-dopamine
[H193K]
[H193K;E396H]
Wt
-9
-8
-7
-6
-5
-4
-3
-2
Log [Zn-bipyridine]

Fig. 9

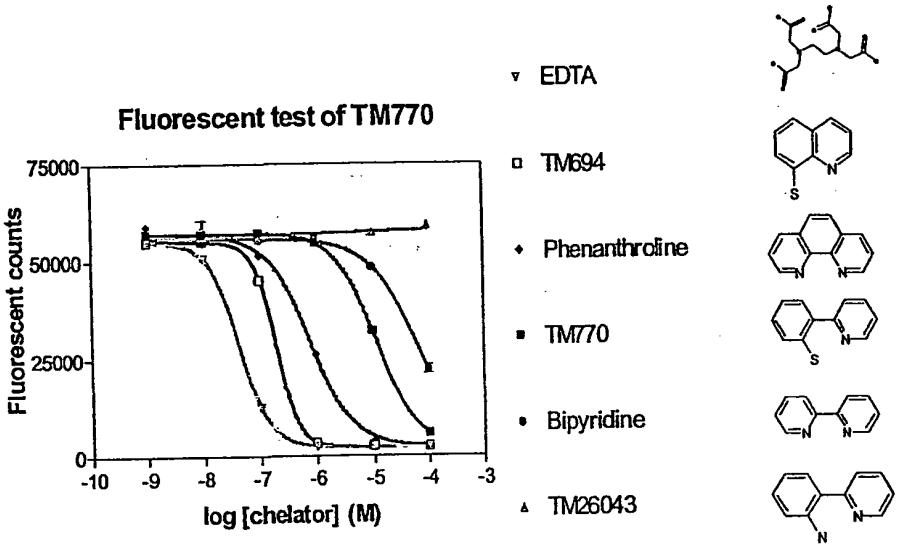

Fluorescent test of TM770
Fluorescent counts
75000
50000
25000
0
-10
-9
-8
-7
-6
-5
-4
-3
log [chelator] (M)
EDTA
TM694
Phenanthroline
TM770
Bipyridine
TM26043

Fig. 10

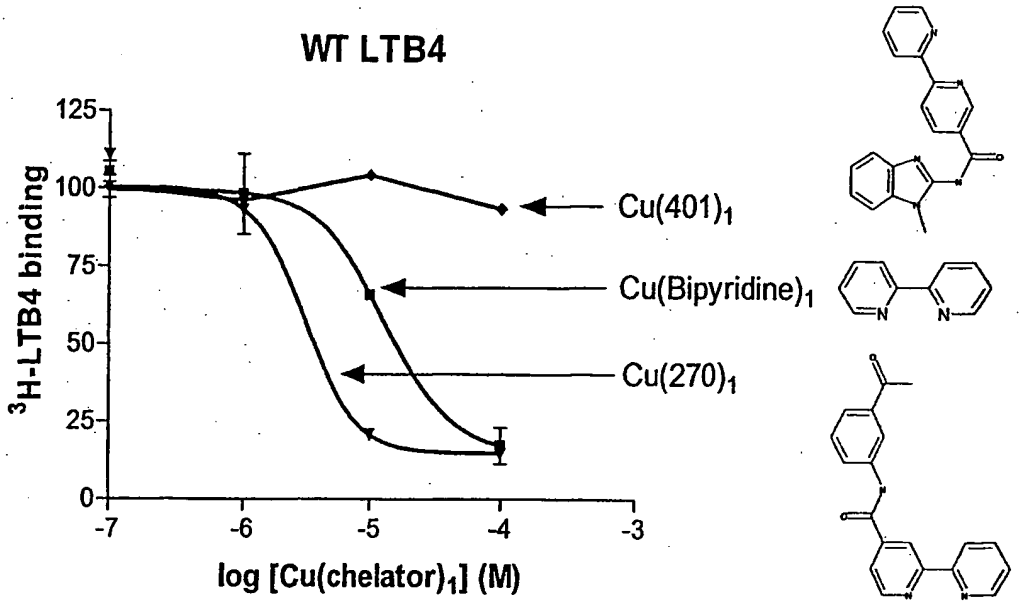
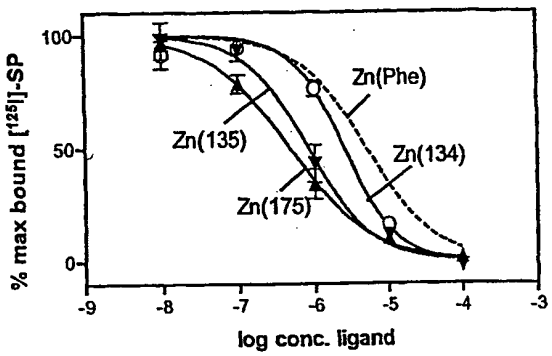
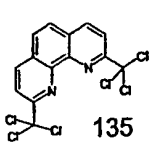
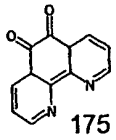

WT LTB4
125
100
75
50
25
0
^{3}H-LTB4 binding
-7
-6
-5
-4
-3
log [Cu(chelator)$_1$] (M)
Cu(401)$_1$
Cu(Bipyridine)$_1$
Cu(270)$_1$

Fig. 11

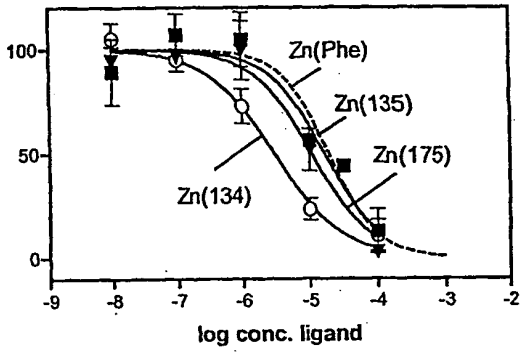
A.


[HisV:05;HisVI:24]-NK1
% max bound [125I]-SP
100
50
0
-9
-8
-7
-6
-5
-4
-3
log conc. ligand
Zn(Phe)
Zn(135)
Zn(134)
Zn(175)


135


175

B.


[HisIII:08;CysVII:06]-NK1
100
50
0
-9
-8
-7
-6
-5
-4
-3
-2
log conc. ligand
Zn(Phe)
Zn(135)
Zn(175)
Zn(134)

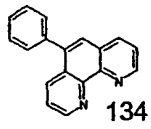
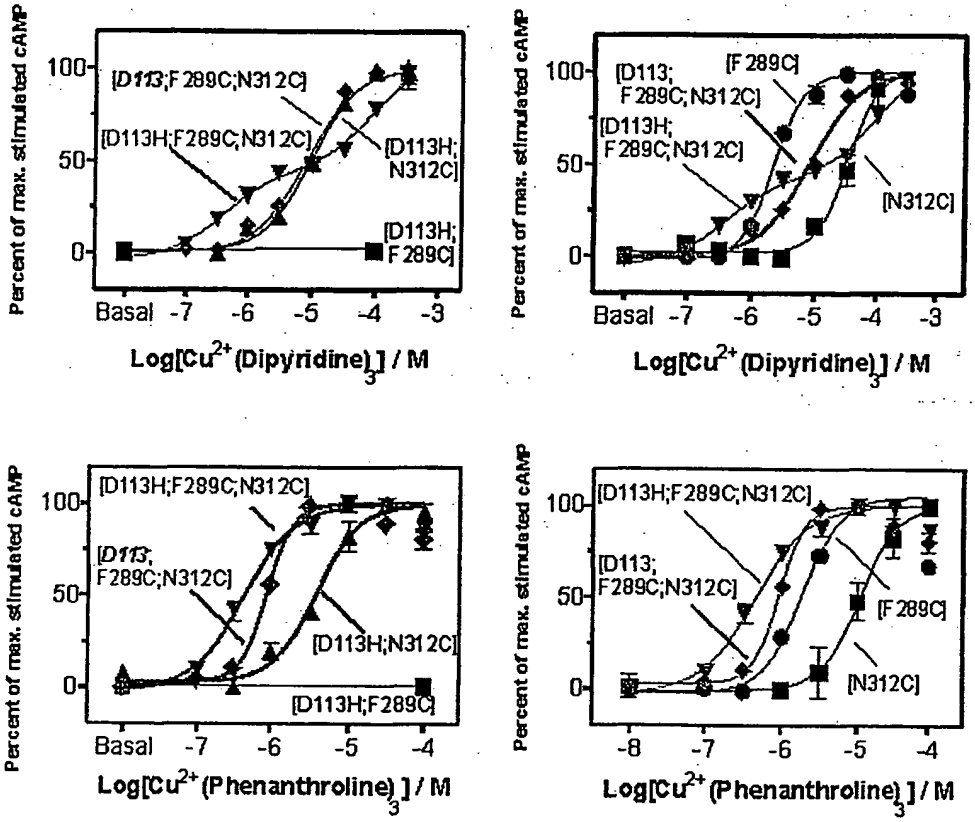

134

Fig. 12

A.


Percent of max. stimulated cAMP
100
50
0
[D113;F289C;N312C]
[D113H;F289C;N312C]
[D113H;N312C]
[D113H;F289C]
Basal -7 -6 -5 -4 -3
Log[Cu2+(Dipyridine)3] / M
[D113;F289C;N312C]
[F289C]
[D113H;F289C;N312C]
[N312C]
[D113H;F289C;N312C]
[D113;F289C;N312C]
[D113H;N312C]
[D113H;F289C]
Basal -7 -6 -5 -4
Log[Cu2+(Phenanthroline)3] / M
[D113H;F289C;N312C]
[D113;F289C;N312C]
[F289C]
[N312C]
-8 -7 -6 -5 -4
Log[Cu2+(Phenanthroline)3] / M

B.

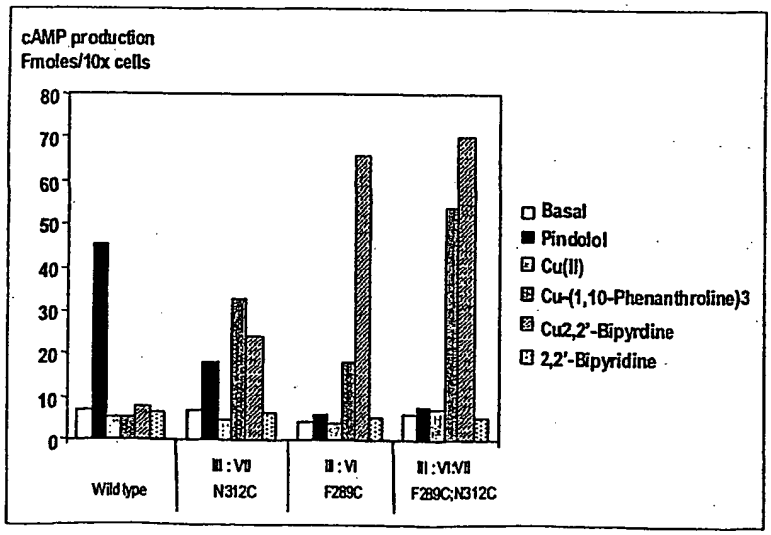
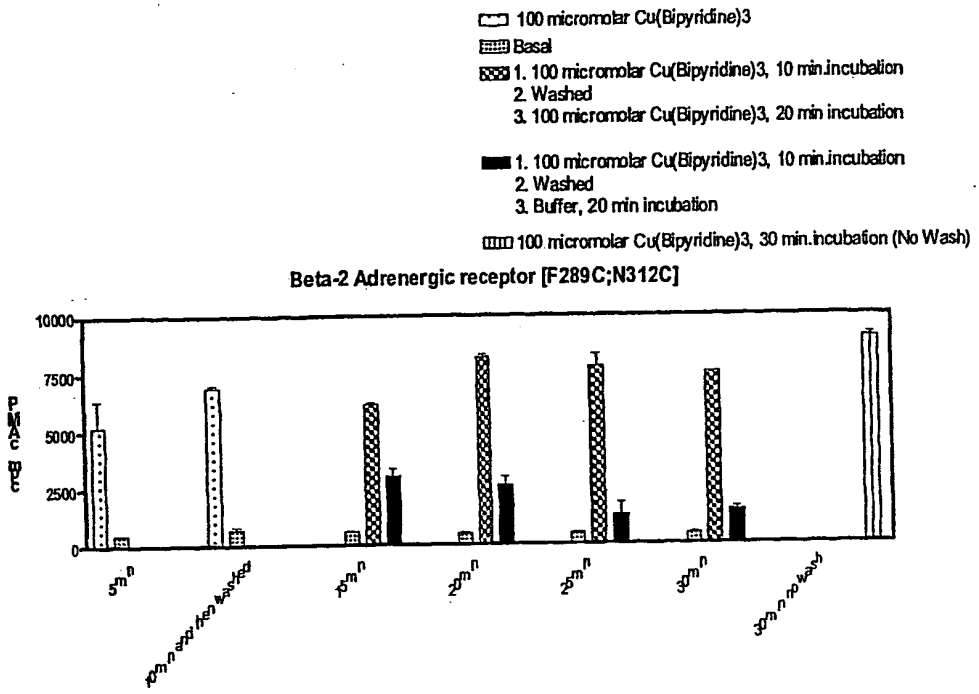

cAMP production
Fmoles/10x cells
80
70
60
50
40
30
20
10
0
Wild type
III : VII
N312C
III : VI
F289C
III : VI:VII
F289C;N312C
Basal
Pindolol
Cu(II)
Cu-(1,10-Phenanthroline)3
Cu2,2'-Bipyrdine
2,2'-Bipyridine

Fig. 13

A.


100 micromolar Cu(Bipyridine)3
Basal
1. 100 micromolar Cu(Bipyridine)3, 10 min.incubation
2. Washed
3. 100 micromolar Cu(Bipyridine)3, 20 min incubation
1. 100 micromolar Cu(Bipyridine)3, 10 min.incubation
2. Washed
3. Buffer, 20 min incubation
100 micromolar Cu(Bipyridine)3, 30 min.incubation (No Wash)
Beta-2 Adrenergic receptor [F289C;N312C]
10000
7500
5000
2500
0
5min
10min washed
15min
20min
25min
30min
30min no wash

B.

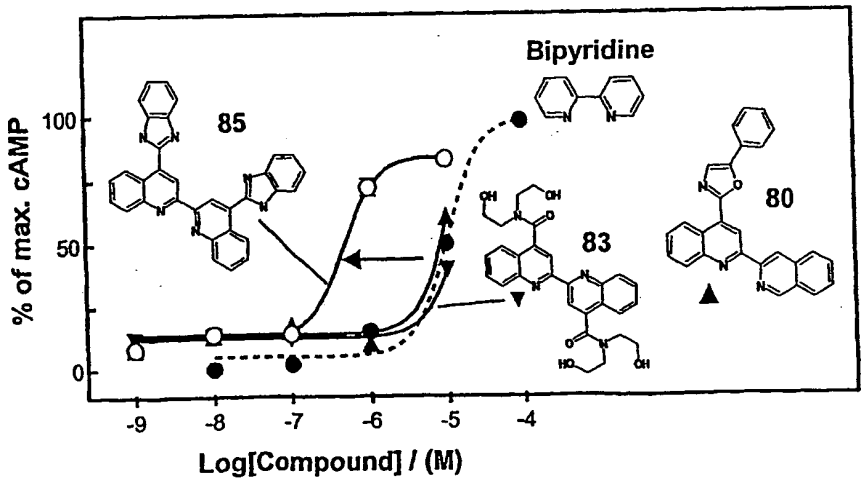

Bipyridine
85
83
80
100
50
0
% of max. cAMP
-9
-8
-7
-6
-5
-4
Log[Compound] / (M)

Fig. 14

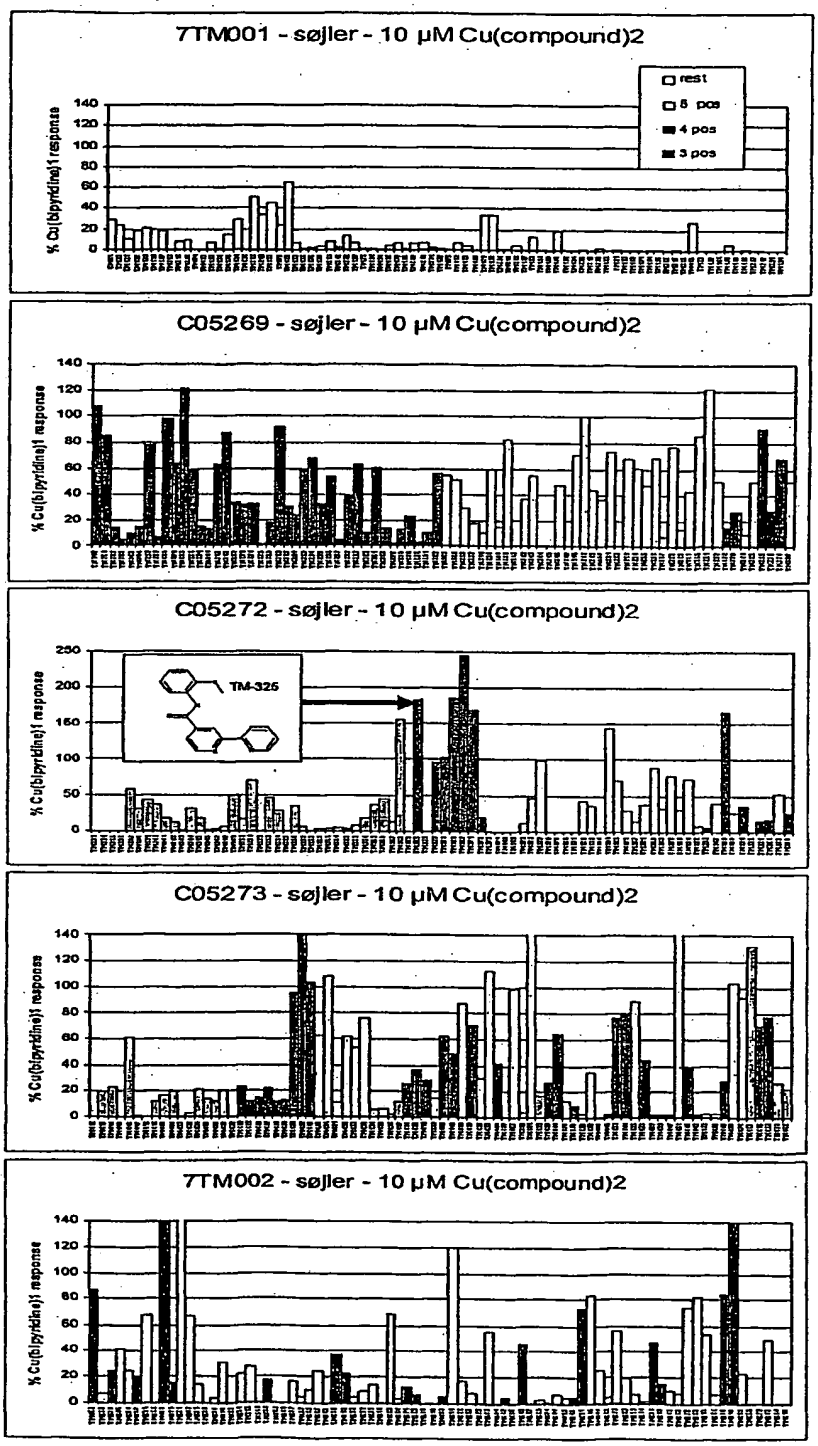

7TM001 - søjler - 10 µM Cu(compound)2
rest
5 pos
4 pos
3 pos
% Cu(bipyridine)1 response
C05269 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response
C05272 - søjler - 10 µM Cu(compound)2
TM-325
% Cu(bipyridine)1 response
C05273 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response
7TM002 - søjler - 10 µM Cu(compound)2
% Cu(bipyridine)1 response

Fig. 15

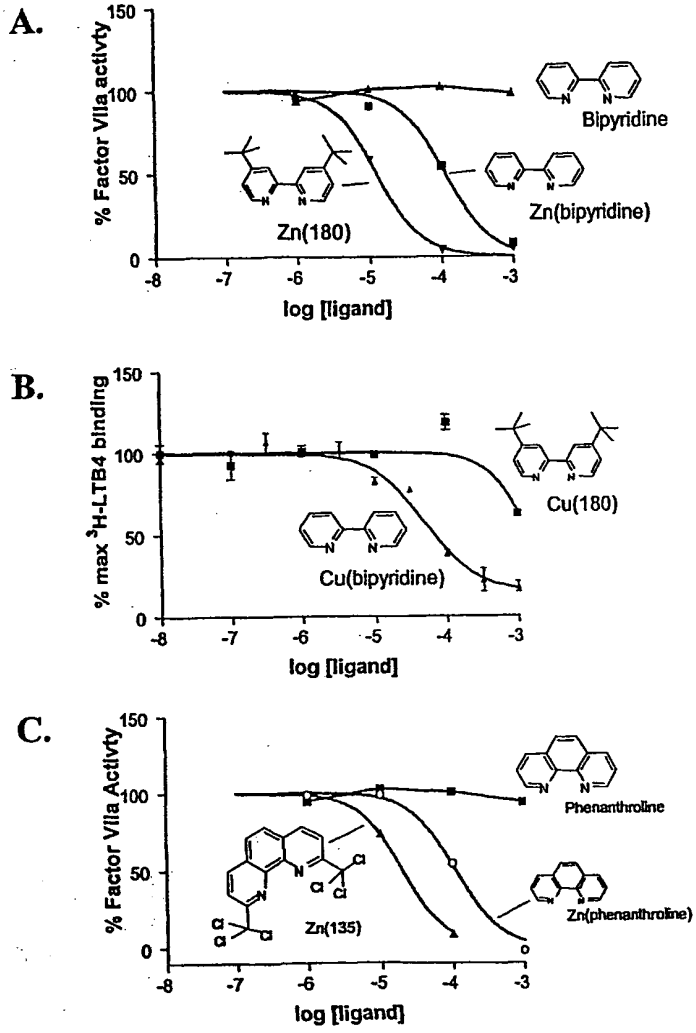

A.
% Factor VIIa activty
150
100
50
0
-8
-7
-6
-5
-4
-3
log [ligand]
Bipyridine
Zn(bipyridine)
Zn(180)
B.
% max ³H-LTB4 binding
Cu(180)
Cu(bipyridine)
log [ligand]
C.
% Factor VIIa Activty
Phenanthroline
Zn(phenanthroline)
Zn(135)
log [ligand]

Fig. 16

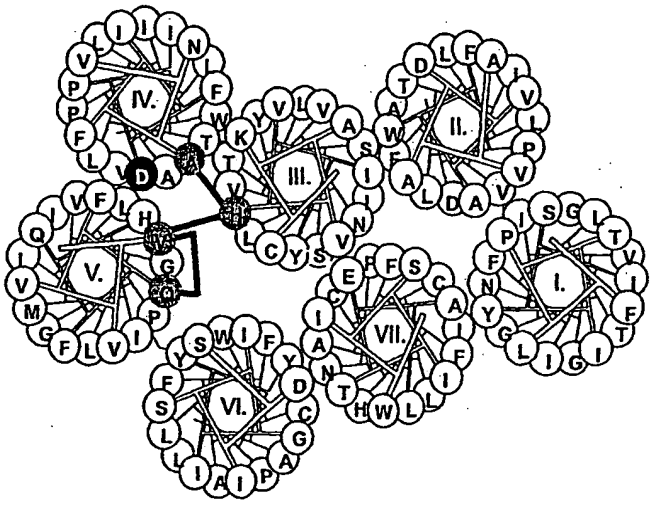
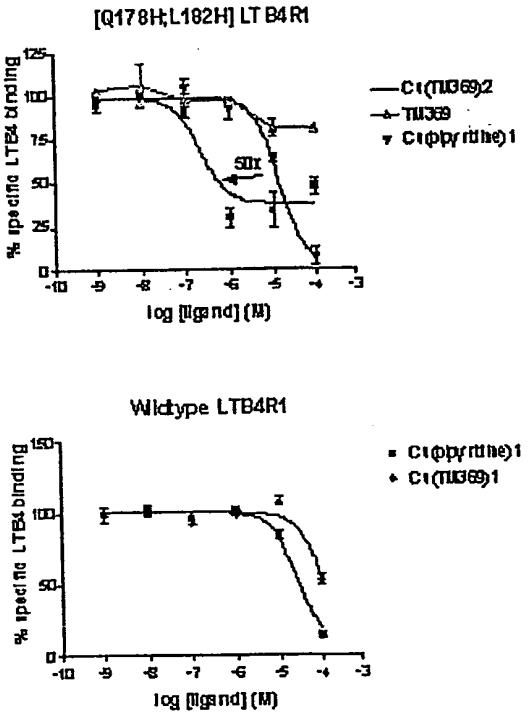
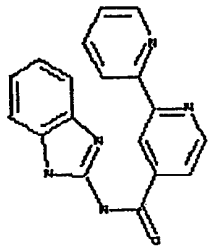


Fig. 17


[Q178H;L182H] LT B4 R1
% specific LTB4 binding
log [ligand] (M)
Ct(TM369)2
TM369
Ct(pyrrhine)1
50%
Wildtype LTB4R1
% specific LTB4 binding
log [ligand] (M)
Ct(pyrrhine)1
Ct(TM369)1



TM-369

Fig. 18

Metal-ion	IC50 on rKAPPA BisHis receptor
Zn(II)	1.1 µM
Co(II)	2.1 µM
Ni(II)	2.7 µM
Cu(II)	4.0 µM
Gd(III)	42 µM
Mn(II)	57 µM

Fig. 19

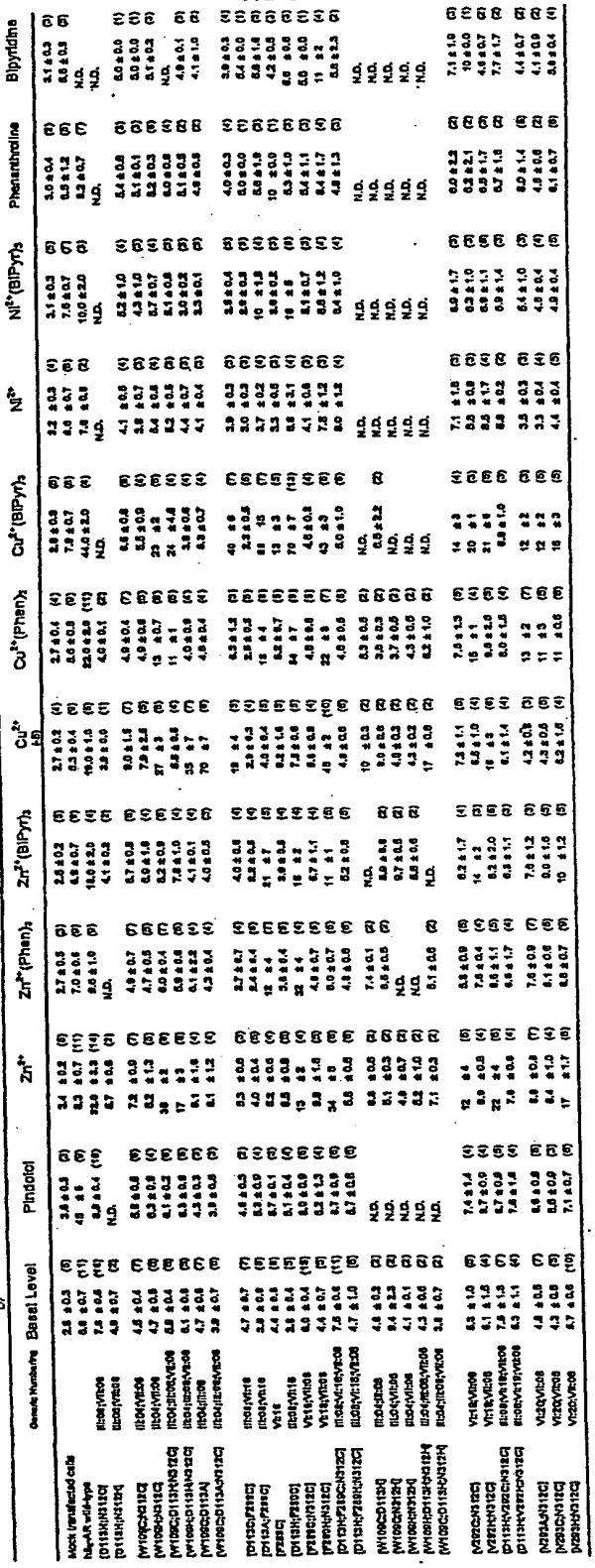
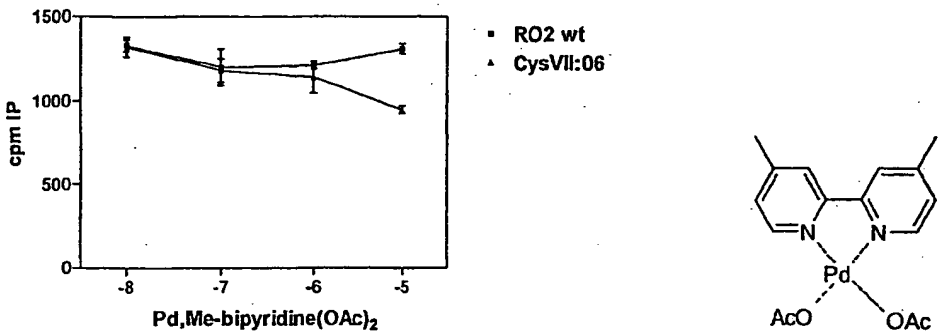


Fig. 20


1500
1000
500
0
cpm IP
-8
-7
-6
-5
Pd,Me-bipyridine(OAc)2
RO2 wt
CysVII:06
N
N
Pd
AcO
OAc

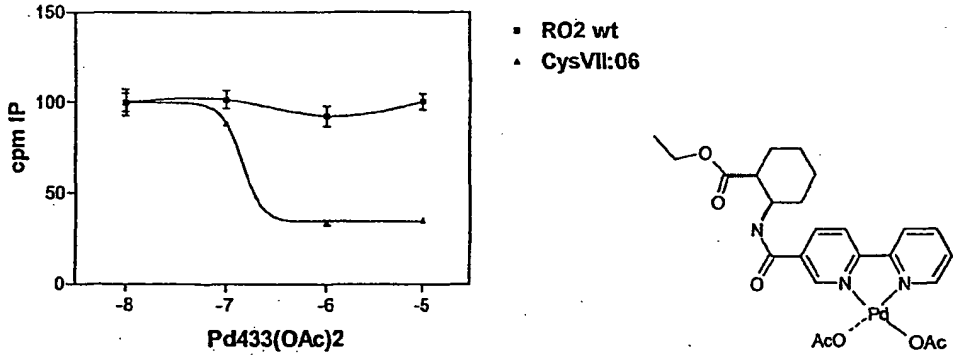

150
100
50
0
cpm IP
-8
-7
-6
-5
Pd433(OAc)2
RO2 wt
CysVII:06
O
O
N
O
N
N
Pd
AcO
OAc

Fig. 21

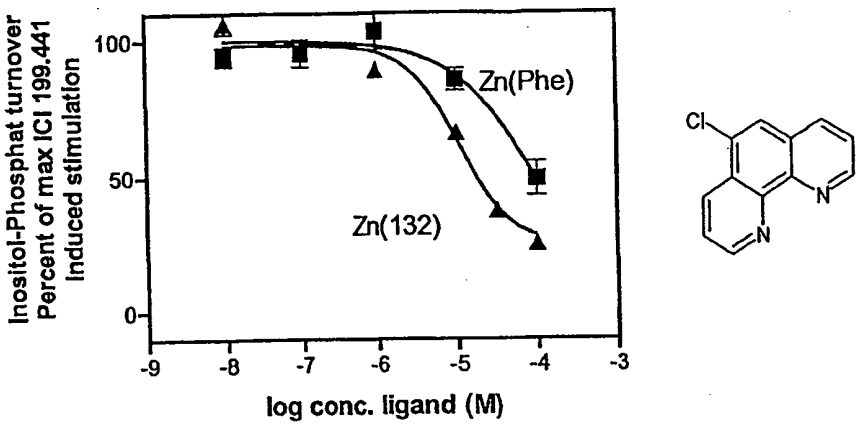

Inositol-Phosphat turnover
Percent of max ICI 199.441
Induced stimulation
100
50
0
-9
-8
-7
-6
-5
-4
-3
log conc. ligand (M)
Zn(Phe)
Zn(132)
Cl
N
N

Fig. 22

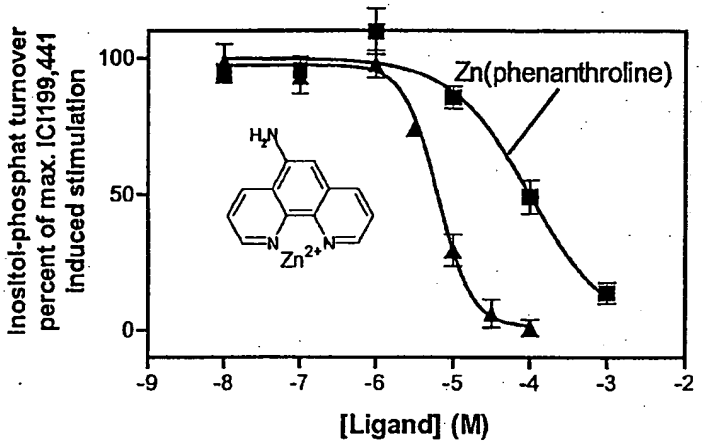

Inositol-phosphat turnover
percent of max. IC199,441
induced stimulation
100
50
0
-9
-8
-7
-6
-5
-4
-3
-2
[Ligand] (M)
Zn(phenanthroline)
H2N
N
N
Zn2+

Fig. 23

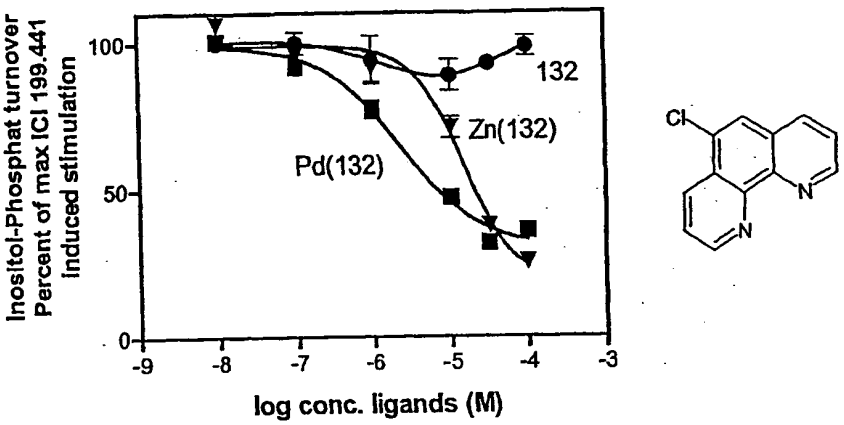

Inositol-Phosphat turnover
Percent of max ICI 199.441
Induced stimulation
100
50
0
-9
-8
-7
-6
-5
-4
-3
log conc. ligands (M)
132
Zn(132)
Pd(132)
Cl
N
N

Fig. 24

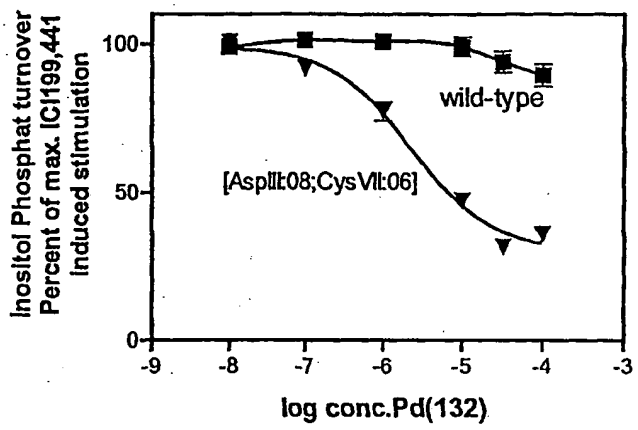

Inositol Phosphat turnover
Percent of max. IC199,441
Induced stimulation
100
50
0
-9
-8
-7
-6
-5
-4
-3
wild-type
[AspIIt08;CysVIt06]
log conc.Pd(132)

Fig. 25

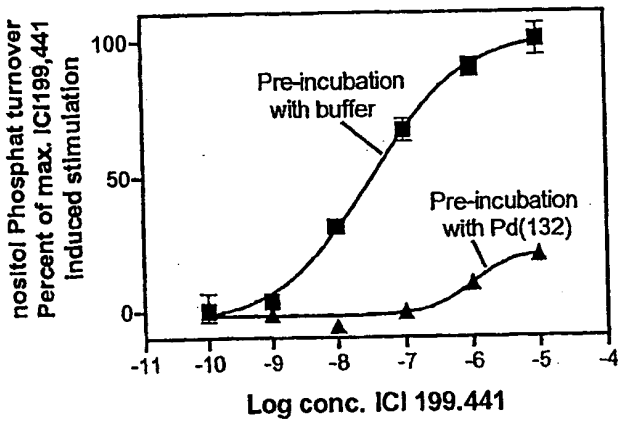

nositol Phosphat turnover
Percent of max. ICI199,441
Induced stimulation
100
50
0
Pre-incubation
with buffer
Pre-incubation
with Pd(132)
-11
-10
-9
-8
-7
-6
-5
-4
Log conc. ICI 199.441

Fig. 26

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 02/00456

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, EMBASE, MEDLINE, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06260 A (CALIFORNIA INSTITUTE OF TECHNOLOGY (US)) 25 January 2001 (2001-01-25) The bipyridines at pages 31-45 and figures 2 and 32,libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method. page 25, line 23 - line 25 abstract	1,2,4, 12,15, 18,28, 31-35

X	WO 98 05961 A (ISIS PHARMACEUTICALS INC (US)) 12 February 1998 (1998-02-12) libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method. page 1, line 17 - line 20; figures 4,10; examples	1-3,7, 32-35

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 2002	07 11 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Per Renström

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 02/00456

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 10016 A (RESOLUTION PHARM INC) 4 March 1999 (1999-03-04) abstract page 1 -page 4 page 30 -page 33; example 18 libraries with metal-ion chelating nitrogen ligands used in a combinatorial drug discovery method, specific metals defined. ---	1,9,23, 32-35
X	SZURDOKI F ET AL: "A combinatorial approach to discover new chelators for optical metal ion sensing." ANALYTICAL CHEMISTRY, vol. 72, no. 21, 1 November 2000 (2000-11-01), pages 5250-5257, XP002902763 page 5254; figure 4 page 5256; figures 6,7 ---	3,8,10, 28,32-35
X	WANG F ET AL: "A Convenient Set of Bidentate Pyridine Ligands for Combinatorial Synthesis" TETRAHEDRON LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 40, no. 26, 25 June 1999 (1999-06-25), pages 4779-4782, XP004168642 ISSN: 0040-4039 page 4780, scheme III page 4781; table 1 ---	1,2,4, 12,15, 28,31-35
X	ELLING CHRISTIAN E ET AL: "Conversion of agonist site to metal-ion chelator site in the beta2-adrenergic receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, no. 22, 26 October 1999 (1999-10-26), pages 12322-12327, XP002174721 ISSN: 0027-8424 abstract; figures 3,4 ---	1,2,5, 7-9, 11-13, 31,33-42
X	ELLING CHRISTIAN E ET AL: "Connectivity and orientation of the seven helical bundle in the tachykinin NK-1 receptor probed by zinc site engineering" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 15, no. 22, 1996, pages 6213-6219, XP002174723 ISSN: 0261-4189 the whole document ---	36-42

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 02/00456

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELLING CHRISTIAN E ET AL: "Disulfide bridge engineering in the tachykinin NK1 receptor" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 39, no. 4, 1 February 2000 (2000-02-01), pages 667-675, XP002174724 ISSN: 0006-2960 page 673, column 2, paragraph 2 -page 674, column 1, paragraph 1 ---	36-42
X	NORREGAARD LENE ET AL: "Delineation of an endogenous zinc-binding site in the human dopamine transporter" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 17, no. 15, 3 August 1998 (1998-08-03), pages 4266-4273, XP002174722 ISSN: 0261-4189 the whole document ---	36-42
P,X	WO 01 50127 A (7TM PHARMA (DK)) 12 July 2001 (2001-07-12) the whole document -----	1-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 02/00456

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-42
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-42

Present claims 1-5, 11, 17-27 and 32-42 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Present claims 36-42 relate to an extremely large number of possible combinations of amino acid sequences defining binding sites. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the combinations claimed, namely the combinations listed in claim 39. Regardless of this however, such lists of combinations are not searchable. Thus, the lists of combinations of amino acid sequences defining binding sites in the present claims 36-42 have not been searched.

The initial phase of the structural part of the search focused on what was considered to be the first apparently searchable part of the invention, namely the invention according to claim 12. However, the search revealed a very large number of documents relevant to the issue of novelty. While being structurally searchable, claim 12 yields so many documents relevant to the issue of novelty upon searching, that it is impossible to determine which parts of claim 12 may be said to define the subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of claim 12 is actually impossible. The same situation was found to prevail also for each of claims 6-10 and 13-16.

Present claim 17 relates to compounds defined by reference to a desirable property, namely that the compounds should be "capable to act as Michael acceptors for SH, -OH and -NH2 groups in the vincinity of the metal-ion binding site". An attempt is made to define the compounds by reference to a result to be achieved. This is not an accepted way of defining compounds, since there may well be compounds having the said property without this being reported, making it impossible to perform a complete search. The expression has thus been ignored.

Present claims 36-42 do not reflect the inventive concept of the target validation process described in the application in a clear and concise way (Article 6 PCT). Furthermore, claims 36-42 are not clear and concise since they relate to "any of items 1-50". Claims should not refer to subsequent claims, and there are not even 50 claims in total.

Present claims 3, 8 and 10 can not be subordinated to claim 1 (see the definition av X-F-Y in claim 1)

What has been performed is basically a broader search not focused on

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

specific structures but instead on, for example, combinations of key terms like combinatorial, libraries, drug discovery, target validation, dentate and chelate, etc.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 02/00456

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0106260 A	25-01-2001	AU 6115300 A	05-02-2001
		WO 0106260 A1	25-01-2001
WO 9805961 A	12-02-1998	US 6077954 A	20-06-2000
		AU 3903697 A	25-02-1998
		WO 9805961 A1	12-02-1998
		US 6329523 B1	11-12-2001
		US 6197965 B1	06-03-2001
WO 9910016 A	04-03-1999	AU 8849398 A	16-03-1999
		CA 2341969 A1	04-03-1999
		WO 9910016 A1	04-03-1999
		EP 1007106 A1	14-06-2000
WO 0150127 A	12-07-2001	AU 2844901 A	16-07-2001
		WO 0150127 A2	12-07-2001
		EP 1242824 A2	25-09-2002
		US 2002061599 A1	23-05-2002